AD NUMBER

AD450639

NEW LIMITATION CHANGE

TO

Approved for public release, distribution unlimited

FROM

Distribution authorized to U.S. Gov't. agencies and their contractors; Administrative/Operational Use; Sep 1964. Other requests shall be referred to Army Biological Labs., Fort Detrick, MD. [_____].

AUTHORITY

BDRL ltr, 27 Sep 1971

TECHNICAL MANUSCRIPT 144

AEROSOL CHALLENGE OF ANIMALS

SEPTEMBER 1964

UNITED STATES ARMY
BIOLOGICAL LABORATORIES
FORT DETRICK

TECHNICAL MANUSCRIPT 144

AEROSOL CHALLENGE OF ANIMALS

SEPTEMBER 1964

UNITED STATES ARMY
BIOLOGICAL LABORATORIES
FORT DETRICK

U.S. ARMY BIOLOGICAL LABORATORIES
Fort Detrick, Frederick, Maryland

TECHNICAL MANUSCRIPT 144

AEROSOL CHALLENGE OF ANIMALS

Joseph V. Jemski

G. Briggs Phillips

September 1964

ACKNOWLEDGMENT

Acknowledgment is gratefully extended to Mrs. D. Jean Maire for her able secretarial assistance and careful thoroughness in the preparation of this manuscript.

ABSTRACT

Experimental uses of artificially created aerosols are manifold and are especially important for respiratory disease studies. Because the respiratory route is often the natural means of infection in man, study of the pathogenesis of diseases in animal counterparts is more meaningful if animals also are infected experimentally by the respiratory route. In contrast to intratracheal or intranasal instillation, challenge with air-borne particulates greatly increases test reliability because variables can be better controlled and quantitated. Dosage, aerosol particle size, aerosol age, environmental temperature, and relative humidity can be controlled, measured, and analyzed to a variable degree. Moreover, additional experimentation can be accomplished to determine host susceptibility to respiratory challenge, estimating or quantitating dose-response curves, testing the effect of therapy or stress, and evaluating the efficiency of aerogenic vaccination. However, respiratory exposure appears to be the least understood challenge method in animal experimentation. This manuscript, therefore, surveys and describes the basic, practical techniques required to challenge animals with infectious aerosols artificially produced. Methods and equipment necessary for the safety of personnel involved in infectious disease work in laboratories and animal rooms also are described.

CONTENTS

TABLES

FIGURES

I. INTRODUCTION

A. HISTORICAL COMMENTS

The recognition and general acceptance of the importance of air-borne infections are evidenced by the contemporary "Conference on Air-Borne Infections" reported in 1961.[1] Although the concept of infection by inhalation has been intermittently propounded in the study of infectious disease epidemiology,[2-5] its significance was first appreciated and realistically understood in the early 1930's.[6]

The question of whether diseases are "air-caused" has had, in past centuries, fervent believers or equally fervent nonbelievers. For example, in antiquity all infections were thought to have originated from "miasma" or bad air. In 1912[7] Chapin stated, "Most diseases are not likely to be dust-borne and they are spray-borne only for two or three feet, a phenomenon which after all resembles contact infection more than it does aerial infection as ordinarily understood...." In a review of air-borne infection, Chope and Smillie (1936)[8] stated, "Air as a factor in the spread of disease is not as important as the early development of the science of bacteriology seemed to indicate." These beliefs were engendered by the absence of adequate technology for aerobiological study, e.g., Chapin used only glass slides to sample aerosols. Nonetheless, six years before Chope and Smillie's publication, Lurie[9] had reported convincing data on the aerogenic transmission of tuberculosis among guinea pigs. Also, Wells (1933,[6] 1934[10]) advanced the theory of droplet nuclei for transmitting infectious agents and developed equipment for quantitatively sampling microbial aerosols.

Renewed interest, based on newer knowledge and more adequate equipment, has stimulated further study of air-borne contagion (Riley, 1961).[11] At the present time the role of air and air-borne material is receiving renewed attention as a causal factor in disease transmission. Studies on the prevention of laboratory infections have provided valuable information, because many of these are probably contracted by the respiratory route. Some important human diseases now considered to be aerogenically transmitted are diphtheria, tuberculosis,[12] pulmonary anthrax,[13] brucellosis,[14] laboratory-acquired infections of tularemia,[15,16] pneumonic plague,[17] pulmonary mycoses such as histoplasmosis[18] and coccidioidomycosis,[19] Q fever,[20] epidemic parotitis influenza,[21] and newer types of viral infection. This list does not include a number of animal pathogens that presumably do not infect man but are responsible for epizootics.

B. AIR TRANSMISSION OF DISEASE

The basic mechanism for transmitting air-borne disease is by droplet nuclei. On occasion resuspended dust particles carrying infectious organisms are implicated. Langmuir[4] described droplet nuclei as small air-suspended

residues arising from the evaporation of droplets emanating from the mouth and nose. These nuclei containing infectious microbes or toxic moieties, make up biological aerosols. Such aerosols are readily produced artificially by spraying or atomizing wet or dried preparations of microorganisms or toxins.

Experimental uses of artificially created aerosols are manifold, but are especially important for respiratory disease studies. Because the respiratory route is often the natural means of infection in man, study of the pathogenesis of diseases in animal counterparts is more meaningful if animals also are infected experimentally by the respiratory route. In contrast to intratracheal or intranasal instillation, challenge with air-borne particulates greatly increases test reliability because variables can be better controlled and quantitated. Dosage, aerosol particle size, aerosol age, environmental temperature, and relative humidity can be controlled, measured, and analyzed to a variable degree. Moreover, the interplay of these factors can be studied—for example, in relation to viability and virulence.

Disadvantages, however, are inherent in aerobiological experimentation. Artifacts may be introduced by the use of pure cultures propagated on laboratory media. The precise significance of aerosol age on virulence of the air-borne organisms is not fully known. Finally, respiratory doses may not be reliably calculated because the degree of lung retention of inhaled aerosol particles, while predictable, usually is not measured.

The many types of experiments involving aerosol challenge of animals include determining host susceptibilities, estimating or quantitating dose-response curves, testing the effect of therapy or stress, and evaluating efficacy of aerogenic vaccination. By the experimental generation of microbial aerosols, many public health problems can be studied fruitfully, as exemplified in past investigations of the effectiveness of glycols for air sanitation,[22] of ultraviolet irradiation for air decontamination,[23] of respirators for filtering out infectious aerosols,[24] and as a means for studying microbiological safety problems in general.[25]

Respiratory exposure appears to be the least understood challenge method in animal experimentation. This chapter, therefore, will endeavor to survey and describe the basic practical techniques required to challenge animals with infectious aerosols artifically produced. Methods and equipment necessary for the safety of personnel in animal rooms and laboratories also will be presented. References will be made to appropriate sources of information on the theoretical aspects of aerosol generation, behavior, and sampling.

II. BASIC AEROSOL TERMINOLOGY

A. BIOLOGICAL AEROSOLS

Aerosol literally means a suspension of solid or liquid particles in a gas. If the aerosol is generated from a wet suspension (as in a cough or sneeze), rapid dehydration of the air-suspended droplets occurs and forms condensation residue or droplet nuclei. Aerosol particles created by atomization of dried or solid material (such as lyophilized material) may be rehydrated to equilibrate with the environment.[26] The term, biological aerosol, usually refers to air-suspended particles containing populations of microorganisms. The term is applicable also to aerosols of microbial toxins and of nonviable antigens administered via the respiratory route.[27]

Regardless of the etiology of a biological aerosol, its air-borne state and eventual fate are governed by both biological and physical action. Biologically, with time, air-borne organisms and toxins can lose their infectivity, virulence, or toxicity in all possible gradients. Physically, air-borne particulates are subject to Stokes' law, which relates the fallout of a small spherical particle to the gravitational law action upon it.[28] This is illustrated in Table I,[29] which shows the settling velocities for various-sized particles. Glassman[30] recommends that the upper limit of microbial aerosols be arbitrarily set at 100 microns. Reagglomeration, coagulation, impingement, and even the effect of electrical space charges, if not controlled, can result in effects on aerosols that preclude efficient and quantitative studies. With "static aerosols," i.e., aerosols that are not generated continuously, physical decay or fallout and effects of aerosol aging can be readily studied. "Dynamic aerosols," on the other hand, which are clouds continuously generated and passed through a tube, allow only newly formed particles to be studied or observed.

TABLE I.[a] SETTLING RATES OF AIR-BORNE PARTICLES[b]

Particles	Size (microns)	Settling Velocity (feet per minute)
Droplets	100.0-400.0	59-498
Dust	10.0-100.0	0.59-59.0
Droplet nuclei	1.0-10.0	0.007-0.59
	0.1-1.0	0.00016-0.007

a. From U.S. Public Health Monograph 60 (1959)
b. For particles of specific gravity 1.0 in air at 70°F

B. AEROSOL CONCENTRATION, RECOVERY, AND DECAY RATE

Methods for assaying aerosols are dictated by the biological nature of the cloud. Estimating the "viable cloud concentration" involves enumerating the quantity of viable organisms in a unit volume of the cloud, usually by plating sampler fluid on solid media and counting the colonies appearing after incubation. Viral and rickettsial aerosols are assayed by inoculating serial dilutions of sampler fluid into appropriate biological hosts; examples are the intracerebral inoculation of mice with encephalitides viruses or the intraperitoneal inoculation of guinea pigs with Q fever rickettsiae. Toxin aerosols can be titrated in animals with potency expressed in an appropriate unit scale, e.g., the expression of the respiratory dose for guinea pigs in terms of mouse intraperitoneal LD_{50}.[31]

In many situations determination of the "per cent recovery" of a microbial aerosol is recommended. For static aerosols, this parameter is the proportion of viable organisms recovered at a particular cloud age in relation to the total viable organisms in the spray suspension. Aerosol per cent recovery is usually calculated when it is desirable to expose animals to predetermined dosages of aerosol or when one wishes to reduce progressively the aerosol concentration to obtain fractional instead of 100 per cent response in a given number of animals. Per cent recovery values provide one way to combine, in a single figure, the efficiency of the disseminator and the resistance of the organism to aerosolization, its subsequent existence as an aerosol, and the contribution of the chamber geometry to total decay.

The stability or "decay rate" of an aerosol is a measure of the loss in concentration of the aerosolized organisms over the period of time the aerosol is studied. The decay rate is usually estimated by calculating the number of air-borne organisms or infectious units per unit air volume at incremental cloud ages. The slope of a curve drawn from the estimated concentration of viable air-borne organisms at various sampling periods is considered as the total decay, and is usually expressed in percentage over unit of time. With microbial aerosols the total decay includes both physical decay (fallout) and biological decay (death rate) of the organisms.[32,33] Physical decay can be calculated by the appropriate assay of fluorescent dyes[34] or of radioactive tagged cells[35] that are incorporated in the slurry to be aerosolized.

Organisms highly stable in the air-borne state, such as Bacillus subtilis var. niger spores,[36] also can be used as physical decay tracers. The difference between the total decay rate and the physical decay rate will be the rate of biological decay.

C. PARTICLE SIZE OF AEROSOLS

Knowledge of particle size is critical in determining the infectivity of an aerosol through inhalation. Particle sizing, or estimation of the size distribution of aerosol particles, is complex and no attempts are made here to detail the problems and methodology. Excellent basic information is available in a book by Orr and DallaValle[37] and in reports by May and Druett,[38] and Pilcher et al.[39]

Studies by Hatch,[40] Landahl,[41] Casarett,[42] Morrow,[43] and others indicate that the critical diameter of particles for deep lung deposition and retention is 1 to 5 microns. Particles larger than five microns may not reach the lung alveoli; those of 0.25-0.5 micron may not be retained. The effects of particle size in aerosol-induced infections have been treated in a review by Goodlow and Leonard;[44] the LD_{50} of air-borne Pasteurella tularensis for guinea pigs increased almost four logs as the particle diameter increased from 1 to 12 microns. Fothergill[45] mentioned six infectious microorganisms for which the guinea pig respiratory LD_{50} is dramatically affected by particle size, and Langmuir[46] discussed this problem. There is little doubt of the increased effectiveness of infectious particles of five microns or less in diameter. However, there are doubts regarding the classical concept that alveolar retention of particles is necessary for infectivity. Evidence has been presented, for example, by McGavran et al[47] and White et al,[48] suggesting that the site of involvement is the respiratory bronchioles and not the alveoli.

A common misconception concerning the size of aerosolized particles results when the physical size of the microbial agent is confused with the sizes of the air-borne particulates in which the agents reside. The characteristics of the aerosol generators, the nature of the suspending fluid, and the environmental temperature and relative humidity are more important determinants of aerosol particle size than is the size of the microbial cells, which determines only the lower limit.

Fortunately, adequate technology is available for producing aerosols of particles predominantly of the critical diameter five microns or less. Two such disseminators are the University of Chicago Toxicity Laboratory atomizer,[49] and the Collison spray device.[50]

With the proper choice of aerosol samplers, some characterization of the particle diameter can be made for a given aerosol cloud. Because the organisms contained in smaller aerosol particles are most likely to reach and be retained in terminal bronchioles and alveoli, it is desirable to sample with a device possessing appropriate particle-size selectivity. A suitable sampler system, which separates an aerosol into one fraction above and one below five microns, has been developed by May and Druett.[38] This pre-impinger type of sampler has a statistical 50 per cent cutoff for particles with diameters equal to or less than five microns. Inhaled doses for animals can be readily

calculated by determining the number of organisms collected in the sampler fluid. Estimates of the aerosol concentration can be expressed only as the number of viable organisms or infectious units contained in particles of five microns or less in diameter. The number of particles containing the live organisms cannot be determined with impinger samplers.

III. AEROSOL TEST FACILITIES AND SYSTEMS

A. STATIC AEROSOL UNITS

An impressive variety of apparatus has been described in the literature for quantitatively studying experimentally produced microbial aerosols. Static aerosol units have been most frequently described, varying from simple to highly complex. Examples of simple systems include those of Griffin et al[51] and Ames and Nungester,[52] who used one- and two-liter glass vessels; the glass chamber of Edward et al;[53] the rectangular metal box of Glover;[54] the 60-liter glass chamber of Loosli et al;[55] and the chambers used earlier by Lange and Keschischian,[56] Martini,[57] and Buechner.[58] The infectious microorganisms used in these chambers include Bacillus anthracis, Mycobacterium tuberculosis, Pasteurella pestis, and influenza virus.

Young et al,[59] Wolochow et al,[60] Albrink and Goodlow,[61] and Pirsch et al[62] are among those who have used cylindrical metal chambers generally described as modified Reyniers-type chambers. One such chamber was described in detail by Rosebury.[49]

Rectangular chambers of various volumes have been employed in studies of experimental air-borne infections.[63,64,65,66,67] Ferry et al[68] ingeniously collected aerosols in balloons to study the effect of aerosol age in relation to viability of aerosols. A quite different chamber design is the rotating drum or toroid developed by Goldberg and his associates.[69] This device has been especially useful for studying small-particle aerosols over long periods of time.

In 1959, Ray[70] described a 115,000-liter cylindrical steel tank used for aerobiological studies. Wolfe[33] reported on similar cylindrical tanks used for quantitative studies of infectious aerosols. He also referred to a 40-foot diameter steel sphere used in carefully controlled experiments to expose volunteer human subjects to biological aerosols. The results of these studies have been reported.[71,72] This uniquely bioengineered sphere, with an ancillary air-processing system to dilute or purge aerosols, has been fruitfully used in aerosol exposure and pathogenesis studies with other animal species. In these studies mice, guinea pigs, rabbits, monkeys, chimpanzees, goats, burros, and birds have been exposed to a variety of infectious microorganisms.

B. DYNAMIC AEROSOL UNITS

The principle of a wind tunnel[73] best describes the operational characteristics of dynamic aerosol systems. An aerosol is generated into a continuous air stream at one end of the equipment and flows past animal exposure ports to or through an aerosol sampler. The best known and most widely

used dynamic system is the aerosol research apparatus or the Henderson apparatus, named after its developer.[74] It consists essentially of a straight tube with diaphragm-fitted ports where animals can be exposed to the aerosols by insertion of their heads (Figure 1).

Use of a modified Henderson exposure tube for holding monkeys, rabbits, and other species being exposed to aerosols of monkey B virus was reported by Chappell.[75] Roessler and Kautter[76] published a description of the apparatus, showing the air flow system and several types of exposure assemblies. There are two commercially available* models of the Henderson apparatus that are identical except for the method of supplying and discharging air. One recirculates air and the other utilizes laboratory service air and vacuum without recirculation. To protect workers from exposure to infectious aerosols, both models have been engineered for use in bacteriological safety cabinets. In the recirculating unit, filters, vacuum pumps, and an air dryer unit are mounted beneath the cabinet containing the apparatus. The nonrecirculating system operates from house air pressure and vacuum lines. The advantage of the nonrecirculating model is that it can be removed from the cabinet when the cabinet is to be used for other purposes.

A scaled-up version of the Henderson apparatus has been developed wherein the aerosol tube is approximately six inches in diameter and six feet long in contrast to the two-inch diameter three-foot tube of the Henderson apparatus. The larger apparatus has been valuable in laboratory studies with large animals. It has been mounted in a laboratory trailer and used to expose human volunteers in vaccine evaluation tests.[77]

Although the operating characteristics of the Henderson apparatus preclude study of aerosol decay and certain other variables, the relatively low cost combined with the simplicity of operation and the degree of quantitation possible makes it the instrument of choice in many studies. Table II shows selected references to the use of apparatus of this type and the animal species and microbial aerosols used. Aerosols are usually generated with a Collison atomizer[50] although other types of aerosol generators have been used.[78] All glass liquid impinger samplers (AGI) are usually used to assess the aerosol concentration. Leif and Krueger[79] used a small version of the Henderson apparatus with animal-holding cups in a larger metal aerosol vessel for infecting animals with air-borne pathogens.

---

* S. Blickman, Inc., Weehawken, N. J.

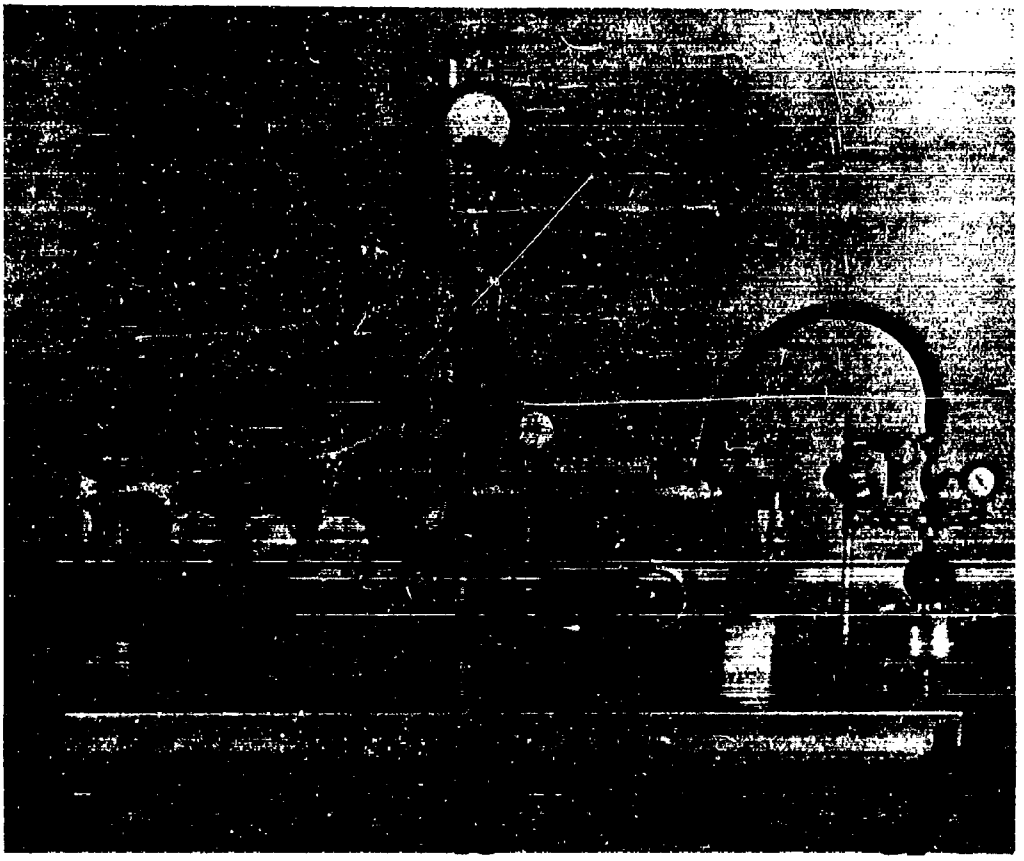


Figure 1. Henderson Apparatus. (FD Neg C-7562)

TABLE II. EXAMPLES OF SPECIES OF ANIMAL AND TYPES OF MICROBIAL AEROSOL USED IN THE HENDERSON APPARATUS

Microbial Aerosol	Animal Species	Source
Bacillus anthracis spores	Rabbits, guinea pigs, mice	Barnes (1947)[181]
Bacillus anthracis spores	Guinea pigs, monkeys	Druett et al (1953)[126]
Bacillus anthracis spores	Monkeys	Henderson et al (1956)[192]
Bacillus anthracis spores	Guinea pigs	Ross (1957)[183]
Brucella suis	Guinea pigs	Elberg and Henderson (1948)[184]
Brucella suis	Guinea pigs	Druett et al (1956)[185]
Brucella suis	Guinea pigs	Harper et al (1958)[32]
Histoplasmosis capsulatum	Guinea pigs	Larsh (1960)[186]
Klebsiella pneumoniae	Mice	Speck et al (1959)[78]
Listeria monocytogenes	Mice, hamsters, guinea pigs, rabbits, monkeys	Kautter et al (1959);[187] Roessler and Kautter (1962)[78]
Monkey B virus	Rabbits, monkeys, guinea pigs, rats, mice	Chappell (1960)[75]
Pasteurella pestis	Guinea pigs	Fukui et al (1957);[127] Lawton et al (1959)[188]
Pasteurella tularensis	Humans	Saslaw et al (1961)[77]
Radioactive spores	Guinea pigs, monkeys	Harper and Morton (1953)[189]

C. BASIC ENGINEERING AND OPERATIONAL REQUIREMENTS

Regardless of the varied configurations of aerosol chambers, if they are to be used productively some basic engineering requirements and principles of operation are applicable to all. Provisions certainly should be made for controlling chamber temperature (ideally by jacketing the vessel) and humidity. Sheckmeister,[80] Kethley et al,[81] and Webb[82] reported on the influence of environmental temperature and humidity in determining the fate or state of microbial aerosols. The number and size of sampling ports and animal exposure ports must be sufficient for the species of animal and the number of aerosol samplers to be used as required by test protocol. Equipment must be designed to conform to the animal exposure method, i.e., animal head, snout, or whole-body exposure.

Interior fanning is required for the static aerosol chambers to prevent stratification of particles, to assure cloud homogeneity, and to secure representative and reproducible cloud samplings. For such chambers, appropriate air processing equipment should be incorporated for diluting aerosols to desired concentrations, or for purging and cleansing the vessel between aerosol trials. Figure 2 illustrates one configuration of a static aerosol chamber. For a dynamic system, such as the Henderson apparatus, the concentration of the aerosol is a function of the concentration of the suspension to be sprayed and the efficiency of the spray device. Dose levels are controlled either by adjusting the concentration of the material to be sprayed or by varying the exposure time.

A primary consideration, independent of the type of aerosol apparatus, is the safe containment of the cloud. This can best be obtained by gas-tight systems with the air pressure within the chamber less than that of the surrounding outside air, or by enclosing the aerosol vessel itself in a ventilated safety cabinet. On larger chambers, peripherally located exposure and sampling ports must either be enclosed in safety cabinets or be so constructed that animals can be exposed to the aerosol without undue risk to the operator. Personnel should wear respiratory protection during the experiments with infectious aerosols.

Infectious effluents from aerosol vessels can be treated by air incineration or by filtration.[83] The vessel, tube, or cabinet interiors can be decontaminated by spraying of formalin with steam and permitting a two-hour contact time.[84] β-propiolactone can be used, but probably is more applicable for decontaminating rooms or buildings.[85] Because of its corrosive nature, peracetic acid is best limited to use with plastic chambers and isolators.[86]

If the liquid effluent from an aerosol vessel exceeds a quantity that can be conveniently and safely collected and sterilized manually, it should be piped to a blow case for pasteurization or steam sterilization before discharge to the sewage system. If spores are not a consideration, treatment of the effluent at 200°F for 30 seconds is sufficient. When sterility is needed, treatment with steam under pressure at 260°F for ten minutes is recommended.

Where animals have been exposed to infectious aerosols, cross-infection hazards to other animals or to caretaker personnel can be prevented or minimized by housing the animals individually in ventilated cages. This problem is discussed in detail in a subsequent portion of this paper.

Basically, the overriding safety requirements are the proper attitude of the administrative and supervisory personnel, and the implementation of written safety regulations and procedures by the operating personnel.[87,88]

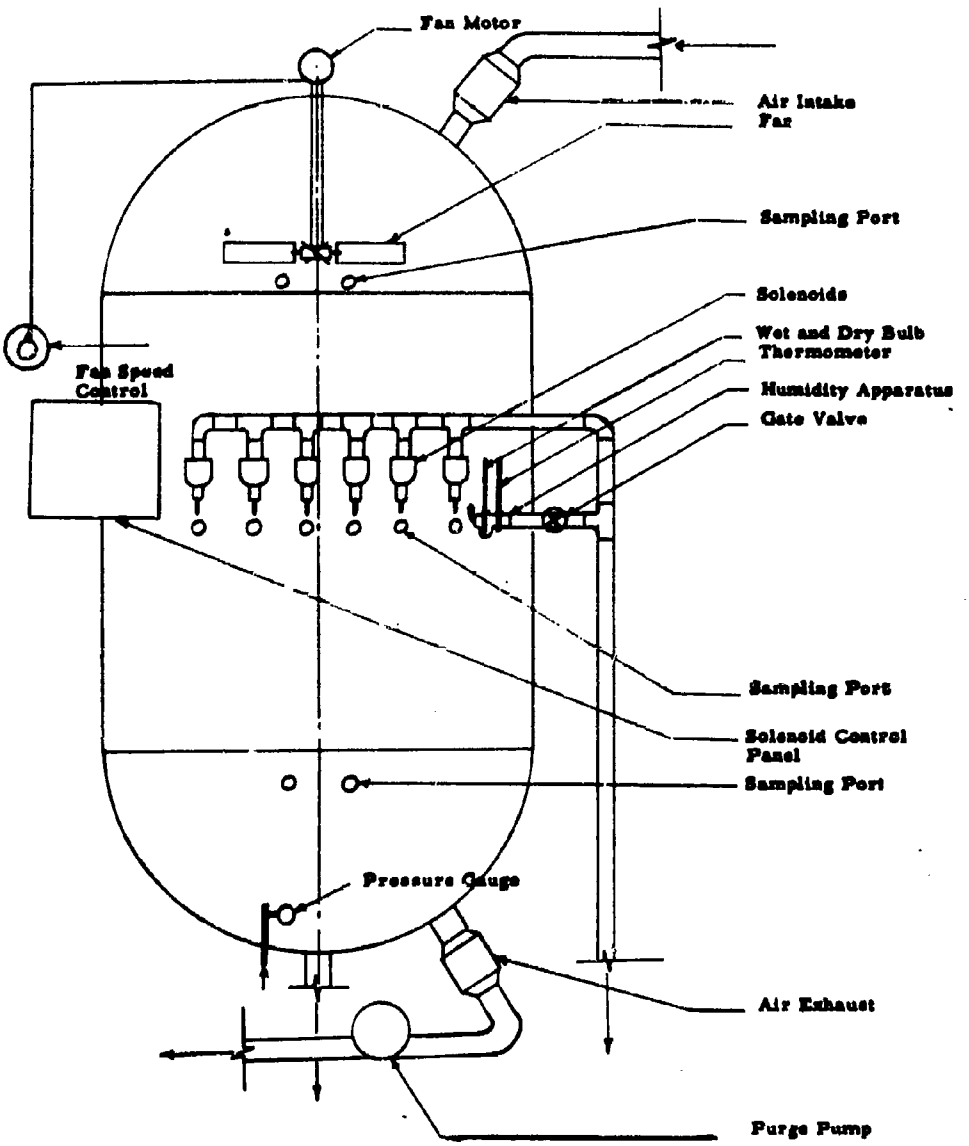


Figure 2. Static Cloud Chamber.

D. RECOMMENDED SYSTEMS FOR ANIMAL EXPERIMENTATION

No particular aerosol chamber system, i.e., static or dynamic, can be recommended as the over-all answer to all investigative aerobiological problems. If the objective is to study only the pathogenesis of experimentally induced air-borne infections, with additional knowledge of the approximate number of organisms inhaled by the animal and a reasonable estimate of the diameter of the aerosol particle containing viable organisms, the Henderson apparatus admirably suits the purpose. A workable safe system for studying infectious aerosols, including the required safety cabinet, probably can be installed for $6000 to $8000. However, only newly formed aerosol particles can be studied in this system. Measures of decay rates, effects of aerosol age on virulence, and relative humidity effects on aerosol stability cannot be studied with this apparatus.

Middlebrook[65] has reported on the use of a 141-liter aerosol chamber during eight years of work on the infectivity and pathogenicity of tubercle bacilli. This apparatus is commercially available* for about $1600.

Wolfe[33] points out that the 500-liter rotating drum is a versatile testing system for investigating small-particle aerosols, and suitable for a variety of experimental objectives. He states it can be procured and installed, with control equipment and housing cabinet, for approximately $35,000.

A large-scale, complex, and expensive aerosol test system such as the cylindrical tank (115,000 liters) with air-process equipment described by Ray[70] would probably cost in the neighborhood of $650,000. This cost includes necessary shake-down and calibration tests. Such equipment, although nearly ideal for studying aerosol-host or dose-response relationships, would be more applicable where the work emphasis is primarily on basic studies of the physical behavior and properties of aerosols.

---

* Tri-R-Instrument Co., Jamaica, N. Y.

IV. GENERATION OF EXPERIMENTAL AEROSOLS

The generation of experimental aerosols for air-borne infection studies requires devices that can produce aerosols of the diameters necessary for penetration and retention at the susceptible sites in the respiratory system. The anatomical site in the upper or lower portion of the respiratory tract not only differs for various diseases, but can be a major determinant for the "infectiveness" of an aerosol. At the level of the lung alveoli, initiation of disease will probably require an aerosol of very small particles, viz., one to five microns. Hatch[89] has reported on the distribution and deposition of inhaled particles in the respiratory tract as a function of aerodynamic principles. The influence of particle size in respiratory infections has been reviewed previously.[44]

A. GENERATION OF AEROSOLS FROM WET SUSPENSIONS

This section describes aerosol disseminators used to generate appropriately sized aerosols for animal challenge. The physics of disseminator configurations and the physical chemistry involved in producing particulate clouds is described in the comprehensive book by Green and Lane.[26] Rosebury's monograph[49] gives specifications on the construction, calibration, and handling of selected glass atomizers used to aerosolize labile agents. Zentner[90] includes a description of specialized disseminators in his report on techniques of aerosol formation.

Atomization of a wet suspension involves dispersion of the liquid, usually by one or more air jets, into small droplets of some particular size. Three general types of atomizers have been employed extensively in aerobiological investigations.

1. Hydraulic-Type Atomizer (One-Fluid System).

With fixtures of this type, the liquid is forced through a restrictive orifice, where it breaks up into droplets. In general, these fixtures are of limited use in infectious aerobiological research.

2. Air-Jet Atomizer (Two-Fluid System).

Most atomizers are based on the two-fluid system, wherein air or other gas jetted at high velocities from an annulus surrounding the fluid flow nozzle mixes with the liquid emerging from the nozzle orifice. The best example of this atomizer type is the University of Chicago Toxicity Laboratories' atomizer (UCTL) as modified and described by Rosebury.[49] The item is all glass, categorized as a peripheral air-jet direct sprayer, and operates by a suction feed to provide fluid flow rates that, depending on the jet diameters, are 0.2 to 8.0 ml per minute. The lower fluid flow rates render this fixture useful in dynamic aerosol systems. More than half the

particles in the aerosol clouds produced by this device have diameters of five microns or less.

Two adaptations of two-fluid nozzle systems have recently been studied. One, termed the FK-8 aerosol gun, has a total liquid volume of 10 ml. The gun will aerosolize, equally well, fill volumes from 1.0 to 10.0 ml. The operating gas pressure is 50 psig, which gives a liquid flow rate of one ml per 3.4 seconds. No residue remains in the reservoir following dissemination. This aerosol gun is useful for small aerosol chamber work and has been used for aerosolization of material in the rotating drum test system.

The second fixture, designated the atomizer test fixture (ATF), is a larger version of the aerosol gun, with a maximum fluid capacity of 40 ml. This fixture will operate at constant efficiency for fill volumes of 1 to 40 ml. As a propellant, the ATF utilizes nitrogen at 75 psig; pressure is exerted directly on the liquid, forcing it through a tube to the nozzle opening. The fluid flow rate is one ml per second or approximately three times faster than that of the FK-8 nozzle. The ATF has absolute scavenging efficiency, i.e., complete dispersion of the liquid fill during dissemination.

Both fixtures are very efficient and produce aerosols in which 50 to 60 per cent of the particulates are five microns or less in diameter after equilibration with the atmosphere. The rapid fluid flow rate of these nozzles makes them usable only in static aerosol systems. Further information on these items is available from our laboratories. The Chicago Atomizer, the FK-8, and the ATF can all be autoclaved.

There are many other variations and modifications of two-fluid atomizers, especially of the DeVilbiss line. However, the items specifically described in the preceding paragraphs are fixtures that have been shown to meet the needs of investigators in aerobiology. These atomizers are highly recommended for studies of experimentally induced air-borne infections in animals.

3. Baffle-Type Atomizer (Nebulizer).

Nebulizers are indirect spray generators that atomize a liquid against the side of the container or against a baffle. The large particles, because of their greater inertia, impinge against the wall or baffle and drop back into the reservoir, thus creating a reflux. Smaller particles are wafted up via an air stream and out of an orifice located above the liquid level. The nebulizer commonly used, especially in the Henderson apparatus, is the Collison spray device.[50] This consists of a spray head with a nozzle tube that dips into a glass jar containing 75 to 150 ml of liquid. This device has been modified to contain volumes of 10 to 20 ml.[76] Compressed air flowing through the nozzle at 26 psig siphons the liquid up through the capillary-sized holes in the base of the spray nozzle. The liquid mass flow rate is about 0.2 ml per minute for nonvolatile liquids. With the Collison

atomizer, 90 per cent of the aerosol reaching the exposure tube is in particles less than five microns in diameter.

Another effective generator is the Vaponephrin atomizer.* It too has a very low fluid flow rate (less than 0.07 ml per minute) and has been used also as a continuous aerosol generator device for dynamic aerosol systems. The primary spray is directed against a baffle, causing a turbulent reflux, with the resulting mist being emitted as an aerosol of very small particles.

Other nebulizing devices, such as those designed by Wells[91] and by Loosli,[92] have been described as efficient disseminators of small-particle aerosols. Although we have had no experiences with these devices, they probably can be used in conjunction with the Henderson apparatus.[93]

B. GENERATION OF AEROSOLS FROM DRY MATERIALS

Dry material or powders are usually atomized by blowing air or gas through the bulk material. The fluidized material is then expelled through an orifice as finely dispersed particulates. The following are examples of suitable laboratory-type devices.

1. Carbon Dioxide Pistol.

A convenient and economical device for the aerosolization of powders is a 22-caliber CO_2 pistol. The muzzle end of the gun is threaded to accept a small hollow tube with restrictive orifices in each end. Both orifices are sealed with aluminum foil diaphragms. The powder to be disseminated (150 to 250 mg) is placed in the back portion of the gun barrel and the sealed adapter tube threaded in place. Upon triggering of the pistol, the CO_2 charge expands into the barrel and entrains the dry fill. When sufficient pressure to burst the primary diaphragm has been achieved, the powder-CO_2 mixture flows into the adapter tube. Further mixing then occurs as the gas pressure continues to build up until the second diaphragm is ruptured, allowing the mixture to escape as an aerosol. In studies with dried preparations (bulk density of 0.3 to 0.4 gm/cc) of Bacillus subtilis var. niger spores, the CO_2 gun produced aerosols with 88 per cent of the viable mass in particles of five-micron diameter or less. The size of the discharge orifice and the diaphragm thickness have been designed to give sonic velocity to the escaping aerosol. These factors probably provide efficient deagglomeration of dry material.

2. Air-Blast Nozzles.

Another simple laboratory disseminator for small quantities of dry powders is a device labeled the OL tube. It consists of two hexagon-shaped

---

* Vaponephrin Co., Upper Darby, Pa.

components screwed together to form a cylinder with a fill volume of approximately two cc. Aluminum foil (0.836-inch thickness) is placed in the base of the container to cover the air orifice and at the top of the container to seal the cavity. The top seal is held in place by a screw cap. After the container is loaded, it is screwed to one end of a hollow tube that is joined to a compressed gas line by a Snap-tite fitting. Application of the gas (nitrogen) at 75 psig breaks the aluminum foil seals and the powder is expelled in aerosol form. The advantage of the OL tube is that it can be easily removed from the pressure line and dismantled for autoclaving, cleaning, and reuse.

A more refined air-blast nozzle for disseminating powders is a two-fluid fixture termed the AP-10 nozzle. This precisely machined item has a constricted inner throat in the nozzle and a button-shaped deflector baffle slightly above the nozzle opening. Fluidized solids fed into the nozzle are forced through the venturi throat and out of the nozzle orifice by air at 40 psig. For more effective dispersion the AP-10 nozzle can be used in combination with a Tornado Feeder device. This feeder has a cylindrical housing that accommodates a smaller cartridge containing the dried powder. The lid of the housing has a centrally located tube (jet nozzle) and two smaller tubes, set at 45 degrees opposing angles to each other, located on either side of the central tube. When the lid is engaged, the three tubes puncture a lead foil cover of the agent container. Nitrogen gas at 135 psig is fed through the two small tubes, creating a tornado-like vortex and causing the powder and gas mixture to be fluidized through the central jet tube into the AP-10 nozzle for further dispersion. The feeder will disseminate 85 to 98 per cent of dry material (such as talc or spores of Bacillus subtilis var. niger) within 30 seconds. The calculated volume of the agent cartridge is 5.92 cc. The Tornado Feeder can be used as a primary aerosol generator or in combination with other nozzles. Further information on the operation of the OL tube, AP-10 nozzle, and Tornado Feeder is available from our laboratories.

C. MISCELLANEOUS DISSEMINATORS

There are many other means and methods for generating aerosols from either wet or dried materials. However, devices such as the vibrating reed[90,94] and the spinning disk of Walton and Prewitt,[95] modified by May,[96] are quite complex and require refined techniques. These items probably are much more applicable for highly specialized aerobiological studies, especially since they are capable of generating particles of uniform size. For the more practical means of challenging animals with infectious or toxic aerosols, especially for pathogenesis studies, the aerosol disseminators described in the preceding paragraphs, in terms of convenience and economy, have proved to be adequate and useful in our experience.

V. SAMPLING BIOLOGICAL AEROSOLS

A. TYPES OF SAMPLING DEVICES

Numerous devices for sampling air to determine its biological or chemical contaminants have been documented. Albrecht,[97] for example, listed 47 air samplers under five categories: (a) sedimentation, (b) centrifugation, (c) electrostatic precipitation, (d) filtration, and (e) fluid bubblers (or liquid impingers). Requirements for air samplers of course vary with the purpose of the investigative effort. Sampling during public health and epidemiological studies requires emphasis on identification of specific organisms among a collected mixed population, with only a moderate requirement for quantitation. Studies of aerosols of known origin used to induce experimental respiratory infections, however, require samplers of high collecting efficiency, especially if the viable organism population of the aerosol is low. Samplers should also be selective in particle acceptance. Only the samplers considered most useful for assessing such aerosols will be described. For a detailed and illustrated review of aerosol samplers, the reader should consult Public Health Monograph 60.[29] An additional survey of samplers has been published by Batchelor.[98]

1. Liquid Impinger Samplers.

Sampling devices that impinge aerosol particulates into or onto collecting fluids are the most frequently used. Low-velocity bubbler samplers are not desirable for small-diameter aerosol particulates because the particles tend to be retained in the air bubbles and escape in the discharged air.[99] High velocity liquid impingers, operating at near sonic air-flow velocities, are very efficient for sampling biological aerosols. The best all-around liquid impinger is the all-glass impinger (AGI), originally developed by Greenburg and Smith[100] and since used in various modified forms.[101,49,33] The AGI (Figure 3) has been described in critical detail by Cown *et al*[102] and evaluated and compared quantitatively with other liquid impingers by Tyler *et al*.[103]

The AGI has a physical particle retention efficiency of approximately 99 per cent for aerosols containing particles of 14 microns or less at a sampling rate of 12.5 liters of air per minute, which is fixed by the orifice size of the capillary fused to the bottom of the inlet tube. The sampler usually is operated with 25 ml of collecting fluid but can be used equally well with volumes as low as five ml. Sampling times are usually one minute, but 5- or 10-minute sampling durations can be used for low-concentration aerosols. Longer sampling intervals result in greater evaporation losses, with possible concomitant increased destruction of the impinged organisms. Foaming can be controlled with a few drops of commercial antifoam agents or sterile olive oil. A feature of the AGI is that most particles with diameters greater than 15 to 17 microns are impinged on the

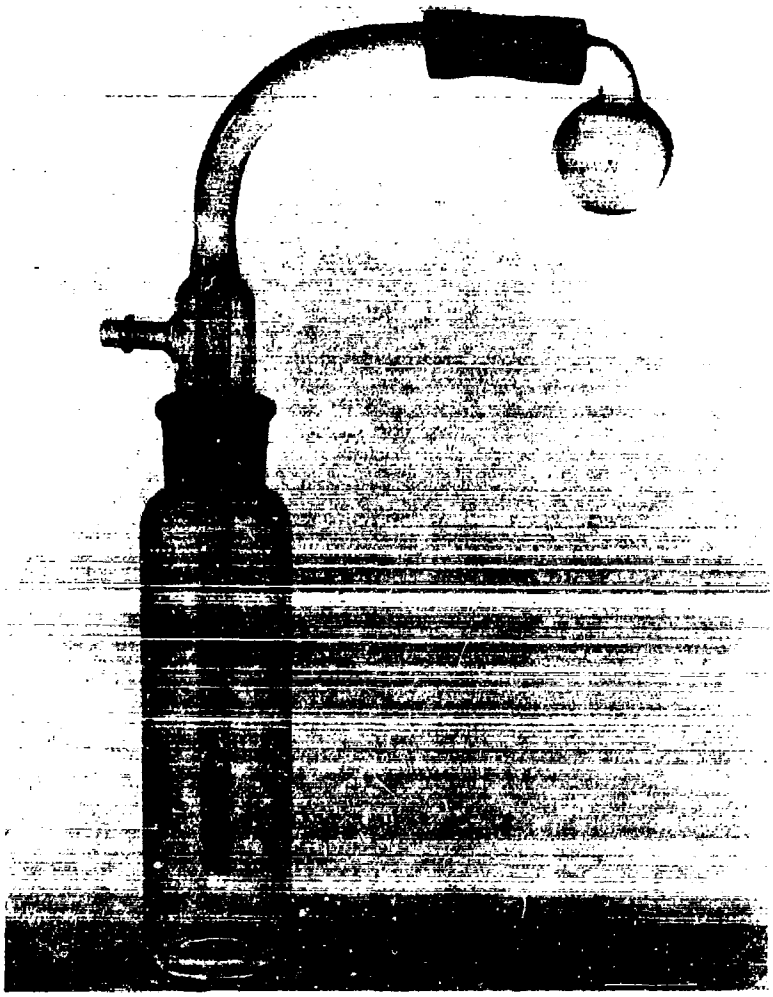


Figure 3. All-Glass Impinger with Pre-Impinger. (FD Neg C-3229)

curved walls of the inlet tube. This discrimination is further enhanced by affixing the May pre-impinger (described in Section II, C) to entrap particles larger than five microns, thus preventing their being passed into the AGI sampler. If quantitation of particles larger than five microns is desired, collecting fluid may be used in the pre-impinger. Assessment of the AGI fluid when the pre-impinger is used provides a good estimation of the viable organisms contained in particles small enough to have reached the lower respiratory tract of mammalian hosts.

The proportion of the total cloud that contains viable organisms in particles of five microns or less can also be measured by use of a glass plate-20 (GP-20) sampler along with the pre-impinger/AGI combination. The GP-20 differs from the AGI in that it has a straight inlet tube and the limiting orifice is a hole drilled through a glass plate at the end of a tube instead of being a drawn capillary. Since the GP-20 does not discriminate among particle sizes, it is considered a total collector. The ratio (x 100) between the aerosol recovery estimated for the AGI and the recovery calculated for the GP-20 will indicate the percentage of particles below five microns in the total cloud that contains viable organisms.

The Shipe sampler is also efficient. Its flow rate (10 liters per minute) is lower than that of the AGI (12.5 liters per minute). The Shipe, instead of having an inlet tube, has a critical orifice in a metal disk cemented to a support tube at the bottom of the flask. The tube is oriented tangentially to both the circumference and the bottom of the flask.[104] This sampler was designed to minimize the cellular damage due to impingement and to eliminate deposition of large particles in an inlet tube.

2. Impactor Samplers.

Impactor samplers deposit aerosol particulates from the sampled air onto solid surfaces. Certain types of impactors are particularly useful when knowledge of the particle size distribution of the aerosol is essential to the experiment.

One of the earliest types, the cascade impactor,[105,106] separates aerosols into four particle size fractions by drawing the air through four compartments, each having successively smaller slits. Particles passing through each slit are impacted selectively on the glass slides. This sampler is useful in air-borne infection studies; not only can the total mass for each slide stage be obtained, but microscopic examination of each slide, if desired, will show the number of particles for each stage.

A more complex type of cascade impactor designed to size and count viable air-borne particles is the Andersen sampler.[107] This device partitions particles into different size ranges by passing the aerosol through five increasingly smaller sieve plates, each located above an agar plate. The sampler operates at a flow rate of one cubic foot per minute. Larger particles

are deposited on the plates in the first stages with the smaller particles appearing in the later stages. After sampling, the agar plates are removed, incubated, and counted to determine the particle size spectrum of the bacterial aerosol. One disadvantage is that superimposition of particles can occur, making the device reliable primarily for low-concentration aerosols.

Mitchell and Pilcher[108] described an impactor with coated slides at each of six collecting stages that could be removed and washed for quantitation of viable organisms. This enables estimation of the median diameter of particles containing viable organisms, a parameter of considerable importance in studies of induced respiratory infections.

A simplified impactor-type sampler used for determining particle size distribution is the single-stage impactor (SSI) developed at Fort Detrick.[109] It is connected in front of the AGI sampler and acts similarly to the pre-impinger as a particle size selector. A family of single-stage impactors have been designed with cutoff points from 1 to 19 microns at 2-micron intervals. The entire family of SSI's or a selected size range of the impactors can be used during an aerosol experiment. Assay of the post-impactor samplers then will give a reasonable estimate of the size distribution of particles containing viable organisms.

A number of air samplers that utilize the impactor principle have been used for environmental sampling. These are typified by the sieve sampler of DuBuy and Crisp[110] and the slit sampler of Decker and Wilson.[111] Except for chamber tests involving very low aerosol concentrations, these samplers are not ordinarily used. They are, however, useful in monitoring the air outside the aerosol chamber for escape of infectious agents.

3. Filtration Samplers.

Filtration samplers are considered total collectors and operate by entrapping particles on a filter bed. The filter bed may be of diverse materials (cf. references in Public Health Monograph 60).[29] Filtration samplers are usually suitable only for collecting spores or other forms, such as viruses, relatively resistant to desiccation. For example, Guyton et al[24] have successfully sampled aerosols of spores with absorbent-cotton collector samplers. After aerosol collection, the cotton is washed in fluid to remove the impinged material and appropriate dilutions of the fluid are assayed. Samplers of this sort will not permit estimation of particle numbers. Aerosols containing Q fever rickettsiae also have been sampled in this manner.[20]

Membrane-type filters[112] are popular for direct microscopic observation and sizing of aerial particles. The membrane filter may also be cultured directly on agar; the growth of colonies on the membrane is an indication of the particles that contain viable organisms.

4. Syringe Samplers.

Several authors have reported the use of syringes for withdrawing samples from small aerosol vessels. Brown and Griffith[113] employed a 100-ml syringe that, when filled with a sample, was discharged into a slit sampler for deposition and culture of the air-borne bacteria.

B. ASSAY METHODS

1. Collecting Media.

The collecting fluids used in liquid impingers must be compatible with the organism or biological material being sampled. The fluids may range from buffered water for spore collecting, to egg yolk-enriched solutions for viral or rickettsial aerosols. Tryptose-saline and gelatin-phosphate solutions are adequate for most aerosols of vegetative bacteria. A recommended all-purpose collecting fluid is a five per cent solution of skim milk solids in distilled water. These liquids also are used for diluting fluids for plating on agar surfaces or for titration in animals. For plating, 0.1 or 0.2 ml of a suitable dilution is placed directly on the surface of an agar plate and spread uniformly with a glass spreader. For animal inoculations, it is necessary to use an appropriate control to assure nontoxicity of the collecting fluid, especially if it is proteinaceous, e.g., brain heart infusion. Antibiotics, crystal violet, actidione, etc., may be used where necessary to control contaminants or to selectively inhibit organisms in a mixed population cloud.

Where solid media are required, as in the Andersen sampler, not only must the agar nutritionally sustain the organisms, but it must be of a density suitable to withstand the shearing forces of the air impingement. Agar concentrations of 1.5-2.0 per cent are usually adequate.

Samples from aerosol samplers should be processed as soon as possible after collection. In our laboratories, samples have been held up to two hours under refrigeration without significantly altering the collected microbial population. Under appropriate refrigeration or freezing, some sampler/organism combinations can be held for more than 24 hours.

2. Sampling Data.

To estimate the number of organisms or infectious units per liter of aerosol, their concentration in the sampler fluid first must be quantitated. This datum is obtained by plating (bacterial aerosols), by titrations in animal hosts or eggs (viral or rickettsial aerosols), or by colorimetric assays (for aerosol tracer dyes or chemical aerosols). Appropriate serial dilutions of the sampler fluid will yield results within acceptable limits of error. The countable range for bacterial aerosol assays, where practical, should be

30 to 300 colonies per plate. Each dilution should be streaked (0.1 to 0.2 ml) on a minimum of four plates. The average plate count from the appropriate dilution level is then used as the basis to compute the concentration of the sampler fluid. Detailed procedures should be established by consulting with a statistician. A general formula for estimating aerosol concentration is:

$$\frac{\text{Concentration per ml sampler fluid x sampler volume (ml)}}{\text{Sampler air flow rate x sampling time}} = \text{Concentration per liter of aerosol}$$

To estimate the per cent recovery of an aerosol in a static chamber, the following general formula can be used:

$$\frac{\text{Concentration per liter of aerosol x volume of aerosol vessel}}{\text{Concentration per ml (gm) of suspension x ml (gm) disseminated}} \times 100 = \text{per cent recovery}$$

For dynamic aerosol systems, a factor equivalent to per cent recovery is referred to as the spray factor, which is the ratio of number of organisms per liter of aerosol to number of organisms per liter of suspension from which the aerosol was produced. This factor is determined by the following formula:

$$\text{Spray factor} = \frac{\text{Concentration per liter of aerosol (a)}}{\text{Concentration per liter of suspension (b)}}$$

$$\text{(a)} = \frac{\text{Average plate count x dilution x volume sampler fluid}}{\text{Total volume of aerosol sampled}}$$

$$\text{(b)} = \text{Average concentration per ml suspension before and after spraying x } 10^3$$

A spray factor must be determined for each suspension sprayed because the viscosity of the fluid affects the rate or efficiency of spray.

C. STERILIZATION OF SAMPLERS

Samplers used in quantitative aerobiology investigations should be cleaned and sterilized before and after use, usually by autoclaving. Liquid impingers and other glass samplers are prepared for autoclaving by plugging the intake and exhaust ports with nonabsorbent cotton. If ethylene oxide sterilization is used, adequate airing of the samplers is required to dissipate the germicidal gases. For samplers that cannot be autoclaved, swabbing with 70 per cent alcohol before use is satisfactory, but exposure to infectious aerosols should then be followed by ethylene oxide sterilization. It is common practice to sterilize empty AGI samplers and add the sterile collecting fluid aseptically just before use. This avoids the problem, when results are tabulated, of attempting to compensate for the amount of sampling fluid lost during autoclaving.

VI. AEROSOL CHALLENGE OF ANIMALS

A. SELECTION OF ANIMALS

1. Sources of Animals.

As in all animal* experimentation, the quality of animals employed in aerobiological research directly affects the validity and reliability of the results. Not only is the researcher faced with the problem of securing the appropriate species of animals in sufficient quantity and at the times needed, but he must also make sure the animals are nutritionally, physiologically, and immunologically suited for the experimentation and are properly conditioned before use.

To match demand with supply, a directory of commercial sources of laboratory animals has been published by the Institute of Laboratory Animal Resources.[114] Commercially available animals obtained from nature are also listed in this compendium. To meet the challenge of providing quality animals, commercial suppliers have organized the Laboratory Animal Breeders Association to promulgate the production of uniformly healthy animals for research.

In selecting or procuring animals for experimentation, consideration must be given not only to the accreditation of the supplier but also to the original habitat of the proposed experimental animal. Obviously, the indigenous microbial and parasitic population of the animals will vary and will depend in part on whether the animals were commercially reared or obtained from nature. Also, in practically all cases, the clinical and immunogenic history will be unknown for feral animals procured for research use.

In our experience with large numbers of cynomolgus monkeys over a number of years, we have observed that the incidence of nodular worm (*Oesophagostomum* spp.) lesions seen on necropsy in these animals may be indicative of their geographical origin. Monkeys obtained from the Philippine Islands seem consistently to have a much higher number of nodules of ecchymotic cysts present on the large intestine than monkeys obtained from Borneo, Sumatra, or Java. Moreover, we rarely have observed lung mites (*Pneumonyssus* spp.) in cynomolgus monkeys, although these acarids or lesions therefrom seem to be present invariably in the lungs of rhesus monkeys.[115,116] It is of interest to note that the section on acariasis in Ruch's book, "Diseases of Laboratory Primates,"[117] makes no specific mention of the cynomolgus ssp. in relation to lung mite infections, but mentions only the rhesus monkey in this respect. Thus, it appears more logical to use the cynomolgus instead of the rhesus monkey for studies involving the respiratory tract, especially in histological

---

* In conducting the research reported here, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

studies of pulmonary changes. Likewise, the susceptible rhesus would be the animal of choice in tuberculosis studies instead of the cynomolgus, which is refractory to this disease. Haberman and Williams[118] found no evidence of active tuberculosis in cynomolgus monkeys held and cared for in the same room with rhesus monkeys, whose infection rate was 16.4 per cent. We, in turn, have observed no tuberculosis in over 4500 cynomolgus monkeys used in our laboratories.

2. Conditioning of Animals.

The reasons why only healthy conditioned animals should be used in research are obvious. Moreover, the methodology and husbandry required to properly condition common types of laboratory animals have been extensively documented. The Animal Care Panel provides professional guidance in problems of this nature. In recent years, the greatly increased use of infrahuman primates has stimulated many excellent reports on either small stable monkey colonies or large dynamic colonies with frequent population changes.[119,123]

Recent emphasis on proper animal care has led to an elevation of animal colonies (location, equipment, and personnel) from a "back room" status to a current and deservedly honored and dignified position. This is evidenced by the greater acceptance of the centralized animal-holding concept and the increased use of veterinarians as animal colony managers. In addition, more and more laboratory animal suppliers describe their product as being free from specific pathogens. Also an increased supply has led to an increased use of germfree or axenic animals, thus ensuring almost perfectly healthy animals, at least in terms of freedom from infectious disease.

Husbandry practices alone, however, will not ensure a suitably conditioned animal if certain precautions are not taken. Of primary concern is the problem of transportation stress. Stress effects have been considered so important that the Institute of Laboratory Animal Resources formed a committee to study the problem. Their efforts culminated in a manual "Laboratory Animals. I. Guddance for Shipments of Small Laboratory Animals."[124] Its usefulness is enhanced by an appendium on purchase or sale contracts that should be of mutual benefit to the animal supplier and user.

Even if transportation stress is controlled, animals must not be experimentally manipulated immediately on receipt. Acclimatization to the new environment is required if reliable response data are to be obtained. Even animals received from an installation animal farm should be held in a laboratory anteroom at least 24 hours or more before actual use. For larger animals, it has been our experience that pretraining or dry runs have significantly contributed to the conduct and the accuracy of the experiment. This has been especially true when breathing rates are to be metered in animals such as goats, burros, and chimpanzees. In our preliminary efforts, excessive salivation occurred in the masks with almost all animals, but after successive practice trials, salivation was no longer a problem and the animals breathed as normally as could be expected during the actual experiments.

There are additional pitfalls that must be avoided in conditioning animals. One is the inadvertent use of antibiotic-containing feed for animals to be used in infectious disease studies. It should be recorded whether animals have been treated with antibiotic or chemotherapeutic agents up to a week before their use. Another problem is the low relative humidity usually prevalent in animal holding rooms, particularly in the cold months of the year. Flynn[125] has reported that the incidence and severity of ringtail in rats were inversely proportional to the relative humidity. In our laboratories, mouse litters survived poorly until the relative humidity was raised and maintained above 40 per cent. Low relative humidity may cause other undesirable effects yet to be evaluated.

Animals used to study postexposure immunological response must be pretitered before exposure. All animals not reared under controlled conditions must be tested; for animals reared under laboratory conditions, only a statistical sample is required.

B. RESPIRATORY CHALLENGE METHODS

1. Intratracheal and Intranasal Routes.

It has often been assumed that intranasal or intratracheal instillation of infectious material in animals simulates the infections naturally acquired by inhalation. Although there are admitted advantages of the intranasal or intratracheal route, these reside more in the simplified methodology required rather than in duplicating the natural portal of entry. Instillation methods do not allow precise estimation of "inhaled dose" based on animal weight and minute respiratory exchange. Moreover, they provide no opportunity for defining the effects of environmental factors on the infecting agent, and obviously also do not allow determination of the effects of different particle sizes in the inoculum.

The introduction into the respiratory system of rather viscous menstrua containing organisms rarely occurs in nature. It must be recognized, moreover, that the relative position of the mouth, trachea, and bronchi can affect the pathogenesis of diseases, particularly those caused by the introduction of particulates containing microorganisms into the respiratory tract. For example, Druett *et al*[126] found that monkeys exposed to clouds of anthrax bacilli contained in particles 12 microns in diameter often developed massive edema of the face and head that persisted until death, indicating a localized infection beginning somewhere in the head. Animals exposed to aerosolized particles smaller than 5 micron did not exhibit this gross edema.

Fukui *et al*[127] reported that virulent *Pasteurella pestis* cells were cleared much more rapidly from lungs of guinea pigs when inhaled as an aerosol than when introduced intratracheally. Speck and Wolochow[128] described an LD_{50} of 20,000 inhaled cells of *P. pestis* for monkeys in contrast to the

intratracheal LD_{50} value of 100 organisms reported by Ehrenkranz and Meyer.[129] Speck and Wolochow attributed this disparity in LD_{50} to a difference in the infectious process that was affected to an unknown degree by the challenge route. A report by Tigertt et al[72] stated that primary Q fever pneumonia was uncommon in animals or man exposed via the respiratory route. However, these authors also cited a paper indicating that primary pulmonary involvement was engendered in man by intranasal instillation of Q fever rickettsiae.

It is apparent that the histogenesis of a pulmonary disease may depend on how the infecting organisms reach the lungs. Jemski et al[130] observed uniform distribution of tubercles in the lungs of rhesus monkeys that had been exposed to aerosols of virulent tubercle bacilli. Widespread involvement of both lungs has been reported in monkeys exposed to aerosols of P. pestis.[128] McCrumb, et al,[131] however, described a more characteristic lobar disease, with the majority of the lung pathology being restricted to one side in monkeys infected by direct instillation of P. pestis. If one considers the angle of the main bronchi branching from the trachea, it is not surprising that there may be a predilection for instilled liquid to lodge or involve only one side of the respiratory tree.

2. Inhalation Exposure Equipment and Technique.

Although study of the pathogenesis of respiratory diseases is more meaningful when the infection is induced through a route similar to that of the naturally acquired disease, use of infectious or toxic aerosols creates hazards that must be controlled. Specifically, transmission of the experimental infection to other animals or to the animal handler or to the experimenter must be prevented.

a. Restraint Cages for Whole-Body Exposure

For exposures of animals in static or dynamic aerosol systems, it is imperative to employ procedures permitting a normal breathing pattern. In whole-body exposures, this can be accomplished by placing an unanesthetized animal in a perforated metal exposure cage just large enough to contain the animal. Each cage is moved into the aerosol chamber as desired without further handling of animals. The largest animals we have exposed in this manner were monkeys.[132] These cages allow for relatively free movement of the animal and make it unnecessary to bind the arms and legs. The open-lattice construction permits good dissipation of body heat, which is especially important if the animals are to be held in the exposure cage for an extended time. Guinea pigs, hamsters, or mice are placed in similar rectangular cages, compartmented by removable partitions to allow exposure of 4 to 12 animals for a given dose. Roessler and Kautter[78] describe a solid box for exposing monkeys in the Henderson system, where only the head of the monkey protrudes for exposure. Many other exposure devices are available, but on the basis of handling, observation, safe containment with minimum

restraint, maximum animal comfort, and economy in material, cost, and fabrication, the expanded metal cage seems to be the most satisfactory.

b. Head- and Nose-Exposure Restraint Equipment.

In general, whole-body exposures are used for static-aerosol facilities, and head- or nose-exposure techniques are more commonly used in the Henderson system. After placement in a restraining tube with only the head protruding, an animal is exposed by thrusting the head or nose through a rubber iris diaphragm covering the exposure port (Figure 4).

However, for all such exposures, the aerosol tube or chamber exposure ports must be housed in ventilated cabinets to provide protection for the operating personnel. As the head is withdrawn through the diaphragm, momentary differential air pressure may allow some aerosol to escape from the chamber. During head-exposure techniques, the animal must be securely restrained and the normality of respiration of such an animal is difficult to assess. Most restraint tubes, slings, or jackets result in body coverage of the animal, requiring caution that the animal does not overheat. Sedation by mild anesthesia or tranquilizers may result in a more regular breathing pattern (but not necessarily normal), and what additional physiological variables are introduced in this manner are not known.

c. Large-Animal Restraint Equipment and Mask Breathing.

With animals too large to place within a chamber, the aerosol must be brought to the animal. In a large aerosol test sphere, cubicles may be constructed for exposing large animals through masks connected directly with the interior of the vessel (Figure 5). We have used this equipment to expose human subjects, goats, burros, and chimpanzees. Albrink and Goodlow[61] used the face mask technique to expose anesthetized chimpanzees to aerosols of anthrax. In our work with large animals, however, we have not found it necessary to use sedation.

Burros and dehorned goats were restrained in a stall-like box by chains and straps. We later found that horned goats were preferred because the horns can be strapped to a crossbar in the box to immobilize the head. A mask (Figure 6) is fitted over the animal's nose and the head is immobilized by restraint straps. The animal is then transported to the exposure cubicle of the sphere. The open intake of the mask is clamped to a valved tube connected to the interior of the aerosol chamber.

A flutter-type valve allows aerosol to be drawn into the mask by the animal's inspirations. When the animal exhales, the flutter-valve closes and the contaminated expired air is directed through a dry gas meter, in which the volume of each expiration is measured, and collected in a plastic bag. At the completion of exposure, the valve connecting the mask to the aerosol line is shut off and another valve opened to the clean air line in

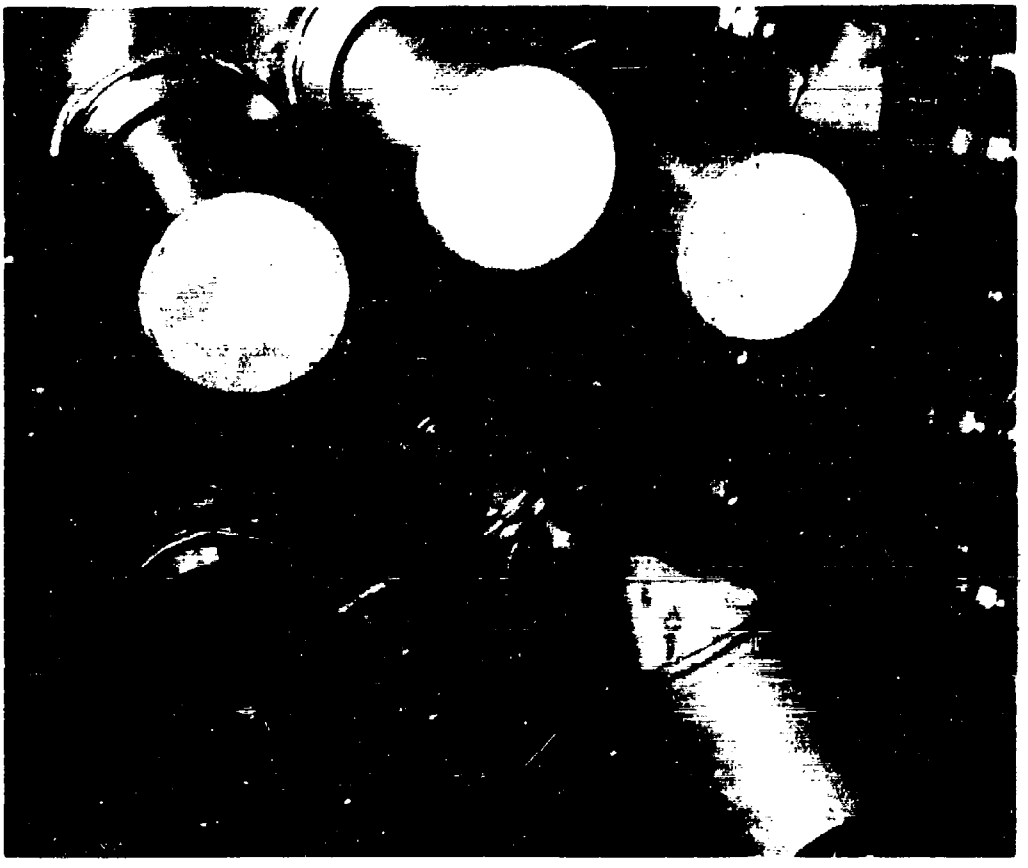


Figure 4. Head Exposure Equipment. (FD Neg C-6480)

Figure 5. Aerosol Exposure Mask Attached Over Nose and Mouth of Burro. (Toweling placed over burro's eyes.) (FD Neg C-7563)

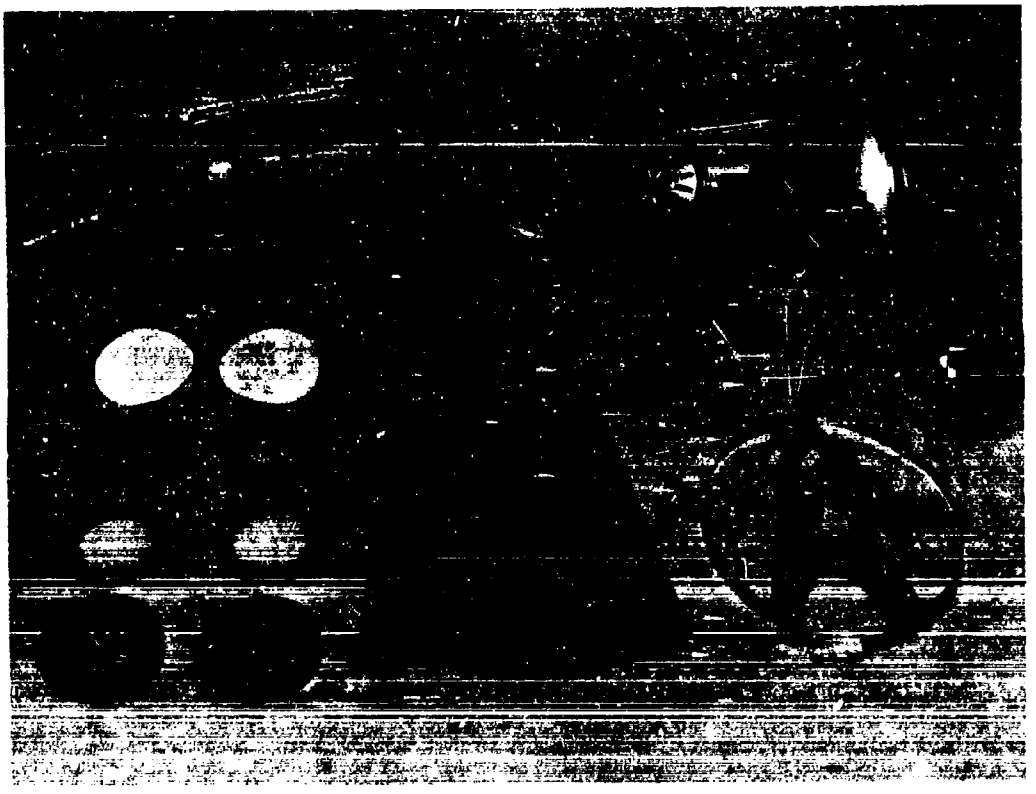


Figure 6. Burro Mask Components. (FD Neg C-7564)

order to flush out the residual contaminated air in the mask. Next, the aerosol intake tube of the mask is unclamped and swabbed down with disinfectant, and another fresh-air intake filter diaphragm is opened on the mask proper. This air filter allows transport of the masked animal to the animal holding area. The safety of these test procedures is attested to by the lack of recovery of aerosol in the cubicle or surrounding area during actual tests.

For exposure of chimpanzees, the animals are placed in a restraint chair adapted from that used at Holloman Air Force Base. The mask, fabricated to cover the nose and mouth of the chimpanzee (Figure 7), is similar to that used for goats and burros. The chimpanzee is then exposed in the cubicle as previously described. This system has been used for animals weighing from 20 pounds (chimpanzees) to 300 pounds (burros). It is our experience that animals must be securely restrained, but with practice runs the animals struggle less and their breathing pattern becomes more regular during exposure.

C. DOSIMETRY

1. Aerosol Dose-Animal Response Relationship

Estimation of animal respiratory doses is dependent on the number of viable organisms per unit volume (usually per liter) of air, respiratory tidal volume of the animal, and duration of exposure. Dose, in this instance, is understood to be the calculated inhaled dose because the degree of retention of the inspired organisms is usually not known and undoubtedly varies even when animals of the same species and equal weight are used. For experimental efficiency and economy of animals, it is important that the aerosol doses be adjusted for fractional responses in groups of exposed animals. The response of the animals, ideally, should be symmetrical around the LD_{50} or ED_{50} (ED = effective dose). The spanning of the desired percentile response is more easily achieved by preliminary titrations of the aerosol in terms of the per cent or number of organisms actually air-borne and their aerostability within the vessel.

Two general methods, which differ somewhat in concept, are used to express dose-response relationships. One is the quantal response which is an "all or none" or "positive or negative" quantitation. Any number of response criteria can be selected, e.g., mortality or survival, presence or absence of febrile reaction, presence of demonstrable serum antibodies, etc. A less commonly used method is referred to as the "graded response" where all (or most) of the animals respond to the given treatment. Survival time, time to onset of fever, and blood sugar level or content are examples of graded-type bio-assays. The usefulness of the two methods has been discussed by Finney[133] and Bliss.[134]

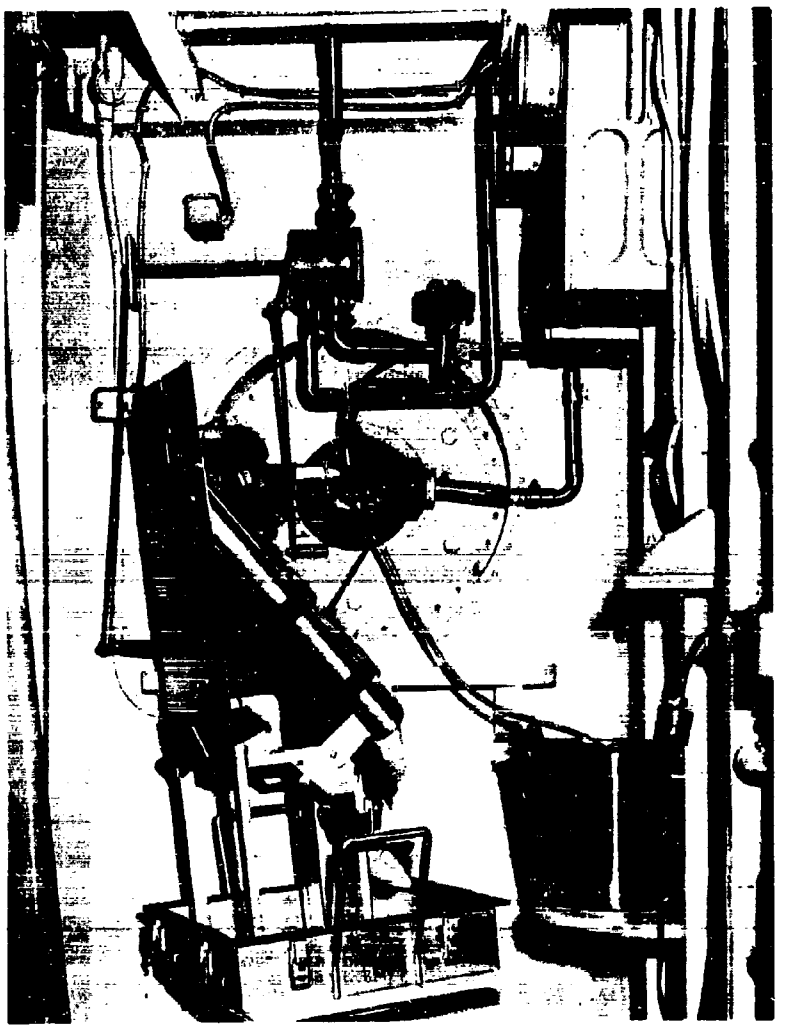


Figure 7. Aerosol Exposure Mask over Nose and Mouth of Chimpanzee and Showing Mask Connection to Aerosol Vessel. (FD Neg C-6959)

2. Experimental Design and Statistics

Any bio-assay procedure should be designed to furnish statistically valid information at the level of precision required or desired. Moreover, the statistical analysis to be used should be stated before beginning the experiment. Cooperative consultation with a statistician for design of experiments is most important, especially if the investigator is to know the degree of precision he may expect from the number of animals, labor hours, etc., he is willing to expend. Also, under- or overdesigning of experiments is minimized if statistical guidance is sought.

The number of animals required for a given level of precision is an ever recurring question. DeArmon and Lincoln[135] state, "A satisfactory experimental design for bio-assay estimates a mean effect, a slope, the degree of slope linearity, and some measure of error."

A nonstatistical but somewhat practical rule-of-thumb guide is that, for an unknown treatment, fewer animals should be placed on more dose points than would be required for treatment for which some information is available. For the latter situation, more animals on fewer numbers of dose points should be used for establishing dose-response curves.

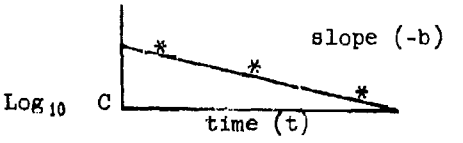
3. Prediction of Aerosol Concentration for Animal Dose

a. Static-Aerosol Chamber System

If the initial aerosol concentration expressed in infectious units per liter of air, and the rate of decay of the cloud are known or predictable, efficient estimates can be made as to when animals should be exposed and length of exposures in order to obtain desired fractional responses. This assumes knowledge of the approximate LD_{50} value of the organism. Aerosol calibration trials, without animals, provide a convenient method of determining the behavorial characteristics for each agent-generator-chamber system. In such trials, the aerosol should be sampled for content of organisms during at least three incremental time periods. The slope of the curve (b value) connecting the observed points is the total decay rate and denotes the rate of decrease in concentration of aerosols with time. For convenience this is often converted to per cent per minute. The slope or b value can be used in the following general formula to compute aerosol recoveries remaining after time t:

$$\log_{10} C_t = \log_{10} C_0 - bt$$

where C_0 is initial concentration, C_t the concentration at time t, and b the decay rate. Graphically, it can be represented as



Values of b for particular decay rates are shown in the tabulation.

Decay Rate (per cent per minute)	b Value (log decrease per minute)
1	-0.0044
2	-0.0088
3	-0.0132
4	-0.0177
5	-0.0222
8	-0.0362
10	-0.0458

The following example illustrates the use of the decay rate or b value to predict the concentration of an aerosol at a given time (30 minutes) following dissemination.

(1) Based on preliminary trials, it has been estimated that: C_0 = initial recovery of 10×10^3 organisms per liter aerosol, b = decay rate of 3 per cent per minute (b - 0.0132).

(2) In the formula $\log C_t = \log C_0 - bt$, we make the appropriate substitution of the estimated values (log concentration at 30 minutes = log initial concentration minus decay rate x 30 minutes post dissemination).

(3) Computation is

$$\begin{aligned} X &= 4.000 - 0.0132 \times 30 \\ &= 4.000 - 0.3960 \\ &= 3.6040 \\ &= \text{antilog } 3.6040 = 4020 \end{aligned}$$

Thus the concentration at 30 minutes = 4020 organisms per liter. This relatively simple method allows estimation of the concentration of aerosol at any point after dissemination so that animals may be efficiently exposed. This computational method also applies to the use of a purge system for rapidly reducing aerosol concentration.

b. Dynamic-Aerosol Systems

In the Henderson apparatus, aerosol concentration is estimated by the use of the spray factor (described in Section IV, B, 2). If it is desired to challenge animals with a particular aerosol dose, the concentration of microorganisms in the suspension to be disseminated is determined as follows:

$$\text{Suspension concentration} = \frac{\text{Infective dose}}{\text{Spray factor}}$$

As in static-aerosol systems, dose predictions are based on prior knowledge of the aerosol characteristics of the organisms under investigation. This knowledge can be obtained only by performing calibration trials to estimate the spray factor.

4. Calculation of Inhaled Dose

a. Estimation of Small-Animal Breathing Rates

The inhaled dose that animals receive can be calculated by the following formula:

Inhaled dose = Concentration of aerosol per liter of exposure time x minute respiratory volume (breathing rate) of animal x exposure duration

The breathing volume of small animals is usually obtained by Guyton's formula.[136] Guyton measured the breathing rates for several species of small laboratory animals and computed a breathing rate factor based on body weight. He also derived a general formula, using a factor representing an average of the animal species tested, that can be used for animals whose respiratory volume has not been calculated. His general formula is:

$$\text{Breathing rate (ml air per minute)} = 2.10 \times (\text{body weight in grams})^{3/4}$$

In this formula, the constant 2.10 is the average breathing rate value. We have found the formula adequate for animals up to 3 to 4 kilograms in weight. For heavier animals it tends to underestimate the breathing volume. A sample calculation of inhaled dose for a 3-kilogram monkey (Guyton's breathing rate factor for monkeys equals 2.35) is:

(a) Breathing rate $= 2.35\ (3000\ \text{gm})^{3/4}$
$= \log 2.35 + 0.75\ (\log 3000)$
$= 974.3$ ml per minute

(b) Inhaled dose = Organisms per liter of aerosol x breathing rate in liters per minute x duration of exposure
= 1000 x 0.9 x 1
= 900 cells

b. Estimation of Large-Animal Breathing Rates

For animals such as dogs, goats, chimpanzees, and burros, the use of animal masks connected to a device for measuring expired air is recommended. This involves measuring the expired air directly by a dry gas meter connected to the mask with a two-way valve system. The mask fits over the animal's face up to, but not including, the eyes. Animals are not placed under anesthesia nor are they tranquilized, but are securely restrained in holding boxes specifically fabricated for the size and species of animal involved. The dry gas meter is calibrated prior to each run.

A comparison of this method of direct measurement of animal breathing rates with estimates derived by Guyton's formula is shown in the accompanying tabulation.

Animal	Weight, lb	Minute respiratory volume, in liters	
		Guyton	Our data
Beagle dogs	26-30	2.3	5.3
Chimpanzees	24	2.3	12.2
Texas Angora goats	70	5.1	13.5
Mexican burros	300	15.0	29.0

The difference in values yielded by the two methods is rather consistent and is the basis for our observation that Guyton's formula may underestimate respiratory exchange rates in larger animals.

Wherever possible, the inspiration of the animal during actual exposure should be metered directly to provide a realistic estimation of the dose received. This refers only to larger species of animal. Fairly accurate dose estimates can be achieved by exposing the animal to a known concentration of aerosol, by controlling the duration of the animal's exposure, and by metering the animal's tidal volume. This method was used in exposing human subjects to low concentrations of aerosols of the rickettsiae of Q fever, the results of which have been reported by Tigertt et al.[77] All human and large-animal exposures were performed by exposing the test subjects through a three-way valved face mask previously described.

5. Aerosol Age in Relation to Aerosol Infectivity

The importance of particle size of aerosols used to induce pulmonary infections has been mentioned previously (Section II, C). It is also important to note that viability of aerosols is often disassociated from virulence. Schlamm[137] has shown that a tenfold increase in the mouse respiratory LD_{50} of P. tularensis resulted from using a challenge aerosol six hours old. Goodlow and Leonard[44] in their review have summarized similar effects, not only for aged aerosols of P. tularensis, but also of P. pestis. We, in turn, have observed that respiratory infectivity of the tularemia organism diminishes appreciably after the cells have been air-borne for two hours or more. Although a number of variables may appear appropriately controlled in experimental airborne infection studies, possible interplay of various factors, such as age and infectivity of the aerosol, should not be overlooked.

VII. MAINTENANCE OF AEROSOL-CHALLENGED ANIMALS

A. TRANSPORT OF ANIMALS FROM AEROSOL EXPOSURE SITES

Safety during work with infectious aerosols depends upon the ability to prevent intimate contact of infecting organism and host. This can be achieved either by the "enclosed system" concept of creating a barrier around the pathogen or by enclosing the host with barriers impermeable to the infectious or toxic material. When animals are exposed to aerosols either bodily or head only, the ideal technique to prevent contamination of the outside environment is to barricade the animal completely during transport to the holding area. Jemski[132] described a method that utilized a transport box with a hermetically sealed interior. The box was ventilated for air-washing the contained animals by attaching a laboratory vacuum line to an air exhaust nozzle on the top of the box. Air entered via an air intake filter port located opposite the exhaust nozzle. In our experiments with goats and burros, the animals are transported to distant holding areas in airtight stalls or chutes. Ventilation is achieved by portable gasoline-motor driven pumps that pull filtered air through and out of the chutes.

Graham and Feenstra[128] developed a transfer box ventilated by a blower that could operate in an automobile. By removing the blower, these boxes were converted for interbuilding transfer to contaminated animals or for transport of animals from pathogen-free rearing areas to user areas.

In our laboratories, animals are removed from the transport box to a five-foot high, stainless steel transfer cabinet[132] containing germicidal ultraviolet lamps. A cabinet originally constructed of marine plywood had also been of value to us in this operation (Figure 8). Air is removed from the cabinet through a glass-fiber filter and discharged into the room air-exhaust duct. This method ensures that cabinet air will not escape into the room environment.

When delivered to the holding rooms, the transport box containing exposed animals is attached to the front of the transfer cabinet. The animals are passed into the cabinet and placed in individual solid-sided cages. The cages are closed with a lid containing air intake and exhaust filter ports, removed from the cabinet, placed on a cage rack, and attached to a vacuum system that exhausts filtered air through the cage at a rate of approximately two cubic feet per minute.

B. ANIMAL CAGING SYSTEMS

1. Experimental Considerations and Cross-Infection Problems

Proper caging of experimentally infected animals is necessary to assure valid experimental results. In practice such experiments are often

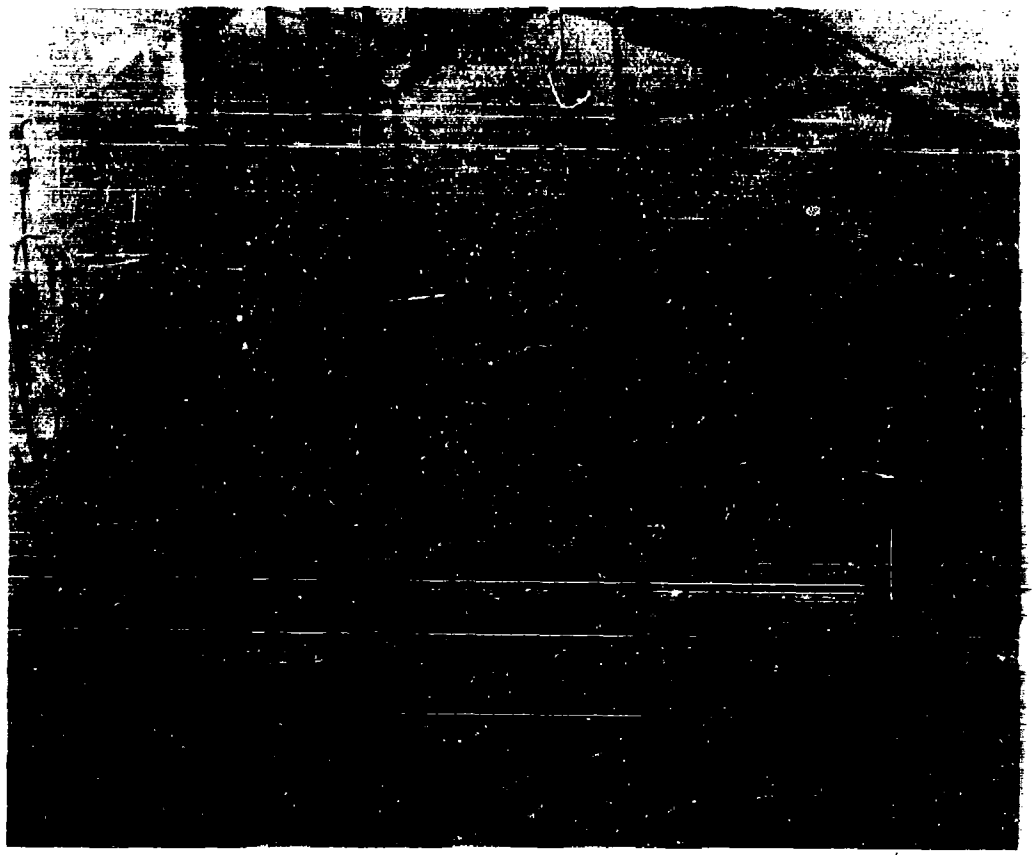


Figure 8. Animal Transfer Hood. (FD Neg C-7565)

complicated by insufficient cages, auxiliary equipment, and labor to carry out recommended procedures. Economics may suggest communal housing of animals, but in infectious disease work undetermined cross-infection resulting from gang caging of animals will seriously impair experimental reliability. Because of cross-infection in cagemate animals, Druett et al[128] applied a correction factor to compensate for the increased infection rate observed during experiments with respiratory anthrax in guinea pigs. In 1956[139] they reported the degree of cross-infection to be dependent on size of the aerosol particles. We have shown the rate of cross-infection in guinea pigs and monkeys, placed as cagemate controls with animals given whole-body exposure to aerosols of anthrax, to be dependent also on the number and species of animals housed in a single cage.[140] In addition, B. anthracis can be recovered from the body, head, feces, and saliva of aerosol-exposed monkeys and their cagemate controls for four days following exposure. This emphasizes the potential hazard to animal handlers when test conditions require daily handling of animals.

Many other documented examples of cross-infection have appeared in the literature. In a selected survey of these, Kirchheimer et al[141] listed the following infectious agents that are transmitted directly between animals during experimental work: Bacillus anthracis, Brucella suis, Eastern equine encephalitis virus, the virus of epidemic diarrhea of infant mice, infectious bronchitis virus, Newcastle disease virus, Pasteurella pestis, poliomyelitis virus, Rickettsia burnetti, Russian spring-summer encephalitis virus, Mycobacterium tuberculosis, Pasteurella tularensis, Venezuelan equine encephalitis virus, and vesicular stomatitis virus. At least half of these agents were transmitted to cagemate controls by animals initially exposed to aerosols.

While precise data are lacking on the magnitude of animal cross-infection with all commonly used infectious agents, with all species of laboratory animals, and under various caging conditions, sufficient evidence is now available to indicate the frequent occurrence of cross-infection. It is sufficient, in fact, to dictate special handling and caging methods for infectious aerobiological experiments. In addition to imperiling the validity of the experiment, these data indicate a probable hazard for men handling aerosol-exposed animals.

Unfortunately, techniques of air-washing aerosol-exposed animals have not proved reliable in eliminating cross-infection. Rosebury[49] concluded that air-washing aerosol-exposed animals for ten minutes reduced coat contamination to a level where there remained "no serious hazard either to the operators or to the animals themselves." Our later experiments[142] showed that guinea pigs bodily exposed for one minute to 490 Brucella suis organisms per liter of aerosol were still able to transmit infection to cagemate animals during the first 24 hours following a one-hour air wash. Moreover, experiments with nonpathogenic spores have shown that aerosol-exposed monkeys continue to contaminate the air of a continuously ventilated cage for as long as nine days following exposure.[140] Guinea pigs tested in the same manner

yielded the bacilli for 18 days.[142] Although air-washing of aerosol-exposed animals is recommended to reduce coat contamination, it obviously should not be depended upon to eliminate cross-infection potential.

Another important hazard relating to the transfer of infection from animal to animal or from animal to laboratory personnel is excretion of infectious forms in urine or feces of the test animals. An incomplete list of diseases in which laboratory animals are known to excrete organisms includes anthrax, brucellosis, cholera, glanders, leptospirosis, lymphocytic choriomeningitis, melioidosis, plague, poliomyelitis, psittacosis, Q fever, salmonellosis, shigellosis, streptococcal infection, tetanus, tuberculosis, and tularemia. Activity of the animals or their caretakers may cause viable organisms in dried feces to become air-borne as a secondary aerosol. The confounding by cross-infection of experimental results has occurred several times in our experience. Animal necropsy has revealed cross-infection between two groups of animals, each inoculated with a different infectious organism and held in nonventilated cages in the same room or air stream. It can be of critical importance to include the proper housing or caging of the test animals in the planning of an investigation of infectious disease in experimental animals.

2. Ventilated Cages and Ultraviolet Cage Rack

It is our practice to cage animals used in aerosol experiments individually in sealed ventilated cages. The ventilated cages we use for monkeys have been described by Jemski.[132] Guinea pigs are housed individually in solid-sided metal cages (9 inches wide, 7 inches high, 11 inches long) closed with a lid that contains a sight glass, an air filter intake, and an air filter exhaust port (Figure 7). Each cage is ventilated by connecting the air exhaust port of the cage to the cage rack air manifold duct with rubber tubing (Figure 9). Because the cage is under negative pressure, cage air does not escape when water bottles are removed for filling. Various investigators have described other ventilated cage systems.[143-146]

To isolate infected animals, some investigators have used ventilated cage racks in which a group of cages is placed in a ventilated device not unlike a glass-fronted book shelf. Another version consists of a ventilated cubicle with sliding doors into which an entire cage rack may be rolled.[147] Although such systems may provide adequate protection for animal handlers, the extent to which cage-to-cage disease transmission is prevented is not known.

A ventilated cage rack used by Lind[148] provides a bank of separately ventilated compartments with sliding plastic doors. Each isolation compartment is designed to receive one cage. Isolation for each compartment is maintained by a small gap at the top of each door through which air enters at the rate of 80 linear feet per minute. In contrast to the system we recommend, Lind's design does not provide for filtration of incoming air.

Figure 9. Guinea Pig Holding Room. (FD Neg C-7566)

Housing animals individually in ventilated metal cages may be prohibitively expensive in many situations. The recent growth of interest in plastic cages and the increasing number of firms developing plastic equipment, however, may reduce costs to a more manageable level. The safety advantages of a ventilated cage system should not be overlooked. It allows the holding of mixed infected animal populations in the same area and permits observation and handling of animals infected with highly communicable disease agents. This has been adequately proved by our years of work, in which we had not one case of occupational illness in personnel directly involved in handling experimental animals maintained in ventilated cage systems.

For areas of lesser risk, a possible substitute for the ventilated cage, and one that maintains isolation and segregation of animals, is the use of open-top cages placed on racks equipped with ultraviolet (UV) lamps. Results showing the efficacy of UV radiation in controlling air-borne infections among experimental animals were reported by Lurie.[149] Phillips et al[150] described a UV cage-rack system that provided a radiation barrier across the top of animal cages. The rack is 5 feet high, 4 feet wide, and 22 inches deep with solid metal shelves. Two 15-watt, 18-inch hot cathode UV lamps with fixtures are needed for each shelf. Each fixture is equipped with a reflector of aluminum to direct the radiation in a band across the tops of the cages. The cages are solid-bottomed and solid-sided to protect animals from the UV radiation. The bottom edge of the lamps is positioned to be level with the top edge of the cages. Tests with this system revealed that the UV barrier effectively reduced the number of air-borne vegetative organisms that escaped from the contaminated fur of the aerosol-exposed animals. The UV barrier also prevented cross-contamination from aerosols of vegetative bacteria produced in adjoining cages, but was only partly effective against bacterial spores. Overhead UV lamps also may be considered in animal holding rooms for use at night to assist in minimizing aerial transport of infectious particles.

The effective use of ultraviolet lamps in controlling air-borne organisms requires understanding and application of three principles: (a) application of sufficient intensity of radiation for an adequate interval of time, (b) proper maintenance to ensure continued output of lamps at the desired intensity, and (c) protection of personnel to prevent radiation burns.

Animal handlers must wear skin and eye protection when working with the UV cage racks. Plastic personnel hoods with fresh air piped into the hood provide this protection. Goggles and respirators can be worn for limited work periods. The UV maintenance program consists of cleaning the lamps frequently and replacing them when intensity measurements indicate 40 per cent loss of output. Intensity meters for UV readings can be purchased from General Electric or the Westinghouse Corporation (Luckiesh-Taylor meter or SM-200 meter).

3. Ventilated Safety Cabinets and Cabinet Systems

The ventilated cabinet provides a means of limiting or controlling incidental or accidental exposure to infectious agents. A variety of ventilated cabinets fabricated from wood, metal, or even plastic is available commercially. Elaborate interconnected systems or modular units have been described by Gremillion[151] and are available commercially. The basic unit is a 34-inch module that can be provided in a one-, two-, or three-tiered height and bolted or glued together to form a continuous system of any length or configuration. Different levels can be interconnected by electrically operated elevators for transfer of animal feed and equipment. All procedures such as injecting, maintaining, and necropsying animals are carried out in the system. It is of interest to note that the modular cabinet system is designed and operated for purposes exactly the reverse of that in gnotobiotic methodology. The modular system, under negative pressure, prevents egress of organisms from within to the outside environment. Cabinets used for gnotobiotic work are maintained under positive pressure to prevent the ingress of organisms from the outside environment. Both systems, however, have the identical objective of preventing accidental infection.

4. Filter Cages

The development of a novel filter cage enabled Kraft[152] to control air-borne spread of the virus of epidemic diarrhea of mice and thus successfully carry out her experiments. The filter cages are cylindrical metal mesh cages with glass fiber insulation material covering the mesh area. Covers fit very snugly and have only one hole to receive the stem of a watering bottle. We have tested cages of this design and found them to be efficient in containing bacterial aerosols. We have also determined that mice will have normal weight gains and be otherwise healthy and apparently comfortable if the amount of filter area on the cage is at least 500 cm^2 for each 100 grams of mice contained within. The insulation also tends to maintain a surprisingly uniform temperature and relative humidity inside the cage. All processing of these cages is performed in a ventilated cabinet provided with a UV entry and exit lock. The filter cages are maintained on cage racks and no cross-contamination has occurred in Kraft's experiments under these conditions. This simple system offers good promise for application in other infectious disease laboratories. However, UV light fixtures on the cage racks are recommended as additional insurance against accidental leaks.

5. Table of Animal Caging Requirements

A summary of recommended animal caging requirements based on method of exposure and the challenge organism is presented in Table III.

TABLE III. CAGING REQUIREMENTS FOR AEROSOL-EXPOSED ANIMALS[a/]

Organisms	Aerosol Exposure Methods	
	Whole Body	Head
Bacillus anthracis	A-10, B	A-5, B
Clostridium botulinum toxin	A-4, C	A-2, C
Brucella species	A-10, B	A-5, B
Coccidioides immitis	A-14, B	A-5, B
Equine encephalitis viruses	A-10, B	A-5, B
Histoplasma capsulatum	A+	A+
Infectious bronchitis virus	A+	A+
Meningopneumonitis virus	A-10, B	A-10, B
Miyagawanella psittaci	A-14, B	A-14, B
Herpesvirus simiae	A+	A+
Mycobacterium tuberculosis	A-30, B	A-30, B
Newcastle disease virus	A-10, B	A-10, B
Pasteurella pestis	A-10, B	B
Pasteurella tularensis	A-10, B	B
Pseudomonas pseudomallei	A-14, B	A-14, B
Rabies virus	A+	A+
Staphylococcus aureus	B	B
Vaccinia virus	B	B
Variola virus	A+	A+
Yellow fever virus	A-10, B	A-5, B

a. A-10 = ventilated cages for 10 days
A-5 = ventilated cages for 5 days
A+ = continuously, etc.
B = open cages in UV racks
C = open cages, no UV rack required

These recommendations are based on existing data and on long experience in caging experimentally infected animals to prevent accidental cross-contamination or infection of animals or handlers.

C. ANIMAL HUSBANDRY PROCEDURES

1. Feeding and Watering Regimens

In a clean area (an area not exposed to infectious organisms), cages to receive aerosol-exposed animals are provided with food and with wood

shavings bedding. In the infectious area, the cages are placed in a cage transfer hood (see Figure 7) where the exposed animal is transferred to a holding cage. The ventilated lid with attached watering bottle is put in position and the entire unit is moved to the cage rack, where it is hooked to the air exhaust manifold.

Guinea pigs are fed daily supplements of kale, which is dropped through the opening used to receive the stopper of the water bottle. Monkeys are fed and watered in pans attached to the inner surface of the ventilated lid (Figure 10). Monkey biscuits are placed in the feed pan by uncapping a small port located on the upper portion of the lid.

Each monkey receives a cube of sugar fortified with two drops of concentrated vitamin B complex liquid each morning, the biscuit pans are half filled in the late morning and afternoon, and a vitamin C supplement is provided by feeding half an orange to each monkey three times weekly. Water pans are filled as required.

2. Animal Inspection and Cage Changing

Animals are observed and checked twice daily on weekdays and once a day on weekends. Since the sight glasses on the ventilated cages tend to restrict visibility, caretakers are instructed to observe movement of animals so that abnormal animals will be detected. Guinea pigs are moved to clean cages twice weekly. All cage changing is performed in the negative pressure transfer cabinet (Figure 7). Clean cages enter from one end of the cabinet and used cages are processed at the other end. The animal is removed from the contaminated cage and placed in a clean one, which is sealed with a lid and replaced on the holding rack. The dirty cage and lid are removed to a large rectangular "boxcar" that is autoclaved when full.

The transfer cabinet may be used for weekly transfer of monkeys to clean cages, but this procedure has been greatly simplified by the use of a monkey transfer tunnel (Figure 11). A clean cage is placed at one end of the tunnel; the cage occupied by the monkey is butted to the opposite end. Two guillotine doors, located near each end of the tunnel, are pulled up and both cage doors are opened, allowing the monkey to scamper through the tunnel into the clean cage. The tunnel serves another purpose, in that the monkey can be entrapped in the center by proper maneuvering of the guillotine doors. Since there is a sight panel on the top and two glove ports with attached leather gloves on either side of the tunnel, restraint of the animal for examination or treatment is simple.

Dead animals are removed from cages in the transfer cabinet. Animal carcasses, with identifying tags, are placed in plastic bags that are closed, dipped in disinfectant solution (Roccal, sodium hypochlorite, or Lysol), and taken to a ventilated cabinet for necropsy.

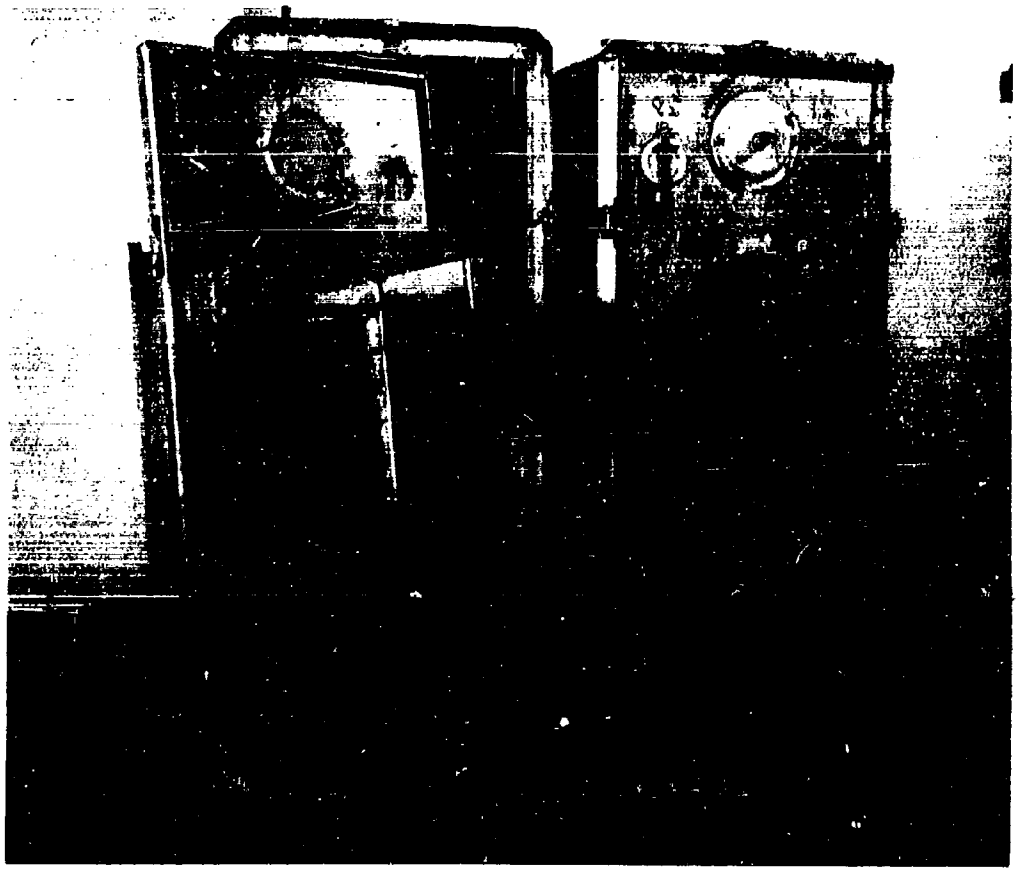


Figure 10. Ventilated Holding Cage for Monkey. (FD Neg C-5738)

Figure 11. Monkey Transfer Tunnel. (FD Neg C-5751)

3. Removal of Contaminated Material from Holding Rooms

Contaminated material from infected-animal holding rooms is placed in containers or packaged to enable the outer surfaces to be sprayed or wiped down with a germicidal solution. The boxcar, containing contaminated cages, is sprayed down before removal to large double-door wall autoclaves (Figure 12) located between the contaminated holding area and the clean preparation room areas. This arrangement provides a positive system for the flow of contaminated discard materials. After sterilization, the boxcar is emptied on the clean side and returned via an autoclave to be reused.

Animal cages, bedding, carcasses, other waste materials, and laundry are autoclaved prior to handling in the clean preparation rooms or prior to being sent out for incineration. A steam autoclave converted for use with ethylene oxide gas mixture is used to sterilize complex or delicate mechanical, electronic, or heat-labile substances that would be destroyed by autoclaving. Details of this method have been reported by Glick et al.[84] The use of low-pressure disposable cans containing a mixture of ethylene oxide and Freon is recommended.[153]

Ventilated lids are processed in the clean area, where all filter pads are changed after each autoclaving. The lids and prepared cages are passed into the animal holding areas via a connecting UV airlock.

For easy cleaning, water bottles and drinking tubes are placed in a tub cage half-filled with water to which one-half cup of Alconox is added. The tub is then autoclaved and the bottles and tubes are removed and rinsed for reuse.

D. SAFETY FOR ANIMAL ROOM PERSONNEL

A safety program for animal caretakers, or any personnel group, should consist of the firm establishment of clearly stated principles of the education of all personnel in safety operations, of immunization of personnel where applicable, and of the proper use of equipment. Most of the procedures and equipment discussed in this section have been proved over many years of tests with highly infectious disease agents.

1. Respiratory Protection: Protective Equipment

A critical device for providing protection to personnel working with animals infected with highly contagious diseases is the ventilated head hood.* All personnel entering infectious disease animal rooms should wear plastic

---

* Snyder Mfg. Co., New Philadelphia, Ohio.

Figure 12. Box-Car and Autoclaves. (FD Neg C-7567)

head hoods supplied with fresh and filtered air by means of flexible air lines (Figures 7 and 10). The hood air line is connected by snap-type connectors to a bacteriological filter canister worn on the animal handler's belt. Air flow can be regulated by a valve on the canister. When the individual leaves the room, the hood line is disconnected and the hood is hung under UV radiation for sanitizing of the outer surfaces.

If ventilated hoods are not available, respirators or gas masks can be used. Some commercial respirators offer adequate protection and have been evaluated by Guyton and Lense.[154] A more recent report[155] describes the effectiveness of five contagion masks. One of the masks is 99 per cent efficient in removing air-borne particles one to five microns in diameter and is reusable after sterilization. Standard military gas masks also provide adequate protection.

Animal handlers should wear surgeon's gloves under veterinary rubber gloves or leather gloves. Wrap-around outer gowns should be worn over laboratory clothing. Before leaving the animal room, shoes and surgeon's gloves should be washed with a liquid disinfectant and the outer gown left in the contaminated area.

All animal rooms should be negative in air balance to the outside corridors. Ultraviolet lamps should be installed around door frames so that a narrow band of radiation screens the opening. Control switches should be placed so that they can be operated by personnel either in the contaminated or outside room area. These lamps should be turned on any time air pressure becomes positive in the animal rooms. As an added safety precaution, they are turned on after the day's work is completed.

2. Operational Safety Regulations and Procedures

A good set of operational safety regulations does not require that every detail of laboratory performance be specified. Rather, it is desirable that the regulations provide the general rules for entrance to and conduct within the infectious unit and guidelines for specific procedures. It is necessary for the regulations to be perfectly clear in the outlawing of known hazardous procedures, such as the oral pipetting of infectious fluids. Moreover, regardless of the size of the animal laboratory, it is recommended that the regulations be written and continually available to operating personnel.

Suggested major headings for "General Biological Safety Regulations" are:

(a) General Building Rules--to include method of building egress and ingress, immunization requirements, clothing, food and drink, smoking, shaving, handling of record books and data sheets, entrance of visitors, etc.

(b) Accident and Illness Procedures--how to secure first aid and medical treatment, accident reports required, etc.

(c) Equipment, Facilities, and Supplies--how repair and maintenance of equipment from infectious units are to be handled; operational requirements for certain equipment; how to remove equipment and supplies; what signs are needed, etc.

(d) Laboratory Techniques--general procedures and precautions, including requirements for the use of cabinets and other safety devices; how to handle spill of infectious cultures, etc.

(e) Animals--use of protective hoods or respirators in animal rooms, care and handling, caging requirements, marking of cages, animal removal, necropsy, disposition of carcasses, etc.

(f) Disinfection and Sterilization--types of liquid and gaseous disinfectants, appropriate concentrations and exposure times, autoclaving procedures, ultraviolet, refuse and sewage disposal, salvage procedures, etc.

To the extent that the use of chemicals or radiological materials presents safety problems, it is sometimes advisable to include sections dealing with these procedures.

3. Vaccination

Vaccination is recommended when a satisfactory immunogenic preparation is available. In infectious disease laboratories, accidental exposure may differ from normal or "public" exposure in at least two important ways: (a) work with infectious aerosols may lead to unusually high exposure dose, and (b) exposure may be by a route foreign to that normally encountered, e.g., respiratory infection with the tularemia or anthrax organism. When there is no vaccine, skin tests before the agent is introduced into the laboratory with periodic follow-ups to determine conversion rates are of value for either prognosis or diagnosis in laboratory epidemiological investigations.

4. Medical Care of Personnel

The role of the physician in the safety program is particularly important when the consequences (morbidity or mortality) of accidental infection are high. Pulmonary anthrax and plague, for example, are rapidly fatal if adequate treatment is not initiated very soon after the appearance of symptoms. The ideal program is one in which personnel are required to report all illness to the physician immediately. Fish and Spendlove[156] and Black et al[157] have discussed the value of pre-employment and periodic medical tests for personnel working in infectious disease institutions.

Desirable medical features of the safety program are: (a) entrance and periodic physical examinations and chest X-rays, (b) administration of vaccines, (c) base-line and periodic serological titers and skin tests of personnel handling certain pathogenic agents, (d) treatment and diagnosis of persons suspected of having occupational illnesses, (e) first aid, (f) free hospitalization for occupational acquired disease, and (g) careful investigation to determine how the infection was acquired. Often, the administration of the accident prevention program, including the analysis of accident records, the formation of safety regulations, etc., is done by the medical department. In any case, the functions of medical and safety personnel should be closely coordinated. It is desirable that the medical officer brief personnel on the potential hazards of any new infectious disease introduced into the research program.

VIII. DIAGNOSTIC PROCEDURES FOR AEROSOL-CHALLENGED ANIMALS

A. USE OF SAFETY CABINETS FOR INFECTIOUS ANIMAL WORK

The most important item of equipment in the prevention of laboratory-acquired infections is the ventilated safety cabinet.[158] The cabinet is a device that not only provides suitable table-top area for the performance of microbiological or necropsy examination, but also provides a physical and transparent barrier (glass shield) between agent and operator. For maximum protection, cabinets should be used for all procedures with infectious substances such as opening culture tubes or plates, blending, tissue grinding, inoculating, injecting, or necropsy of animals, and opening sealed containers of infectious material.

A number of ventilated cabinets suitable for work with infected animals have been described in the literature.[159,160,151,161,25,147,87] Probably the most versatile cabinet is the six-foot stainless steel ventilated model (Figure 13) with a detachable glove panel and UV air locks for entrance and exit of materials.* It should be provided with an air exhaust filter and an exhaust blower that maintains a reduced pressure of 0.5 to 1 inch (water) within the enclosure. Utilities provided in the cabinet should include hot and cold water, vacuum, compressed air, drain, 110-volt a.c. electrical outlet, ultraviolet lamps, and fluorescent lighting. Although cabinets of this type do not provide the gastight absolute barrier features characteristic of the modular cabincts described previously, they do provide adequate protection for most levels of biological risk and are lower in cost than the gastight systems.

Because items removed from a safety cabinet must be autoclaved or decontaminated, it is important that the flow of material in and out of the cabinet be kept to a minimum. Surgical instruments required for necropsy, culture plates and tubes, and tissue-fixative solutions should be placed in the cabinet before work begins. In a given work period, many animals may undergo post-mortem examinations to determine whether death resulted from the infectious aerosols. A systematic procedure must be established, then, to minimize the total number of necropsy instruments required. A satisfactory procedure involves three sets of instruments per prosector with the instruments recycled for each phase of the necropsy by passing through a series of beakers containing water, disinfectant solution, and acetone or alcohol. After the external incision is made, a fresh scalpel is used for further incisions while the initial blade is rotated through the beakers for cleansing and sanitizing. The final acetone rinse is to dry instruments; gas burners are not used in the cabinet. Alcohol burners may be used.

---

* S. Blickman, Inc., Weehawken, N. J.

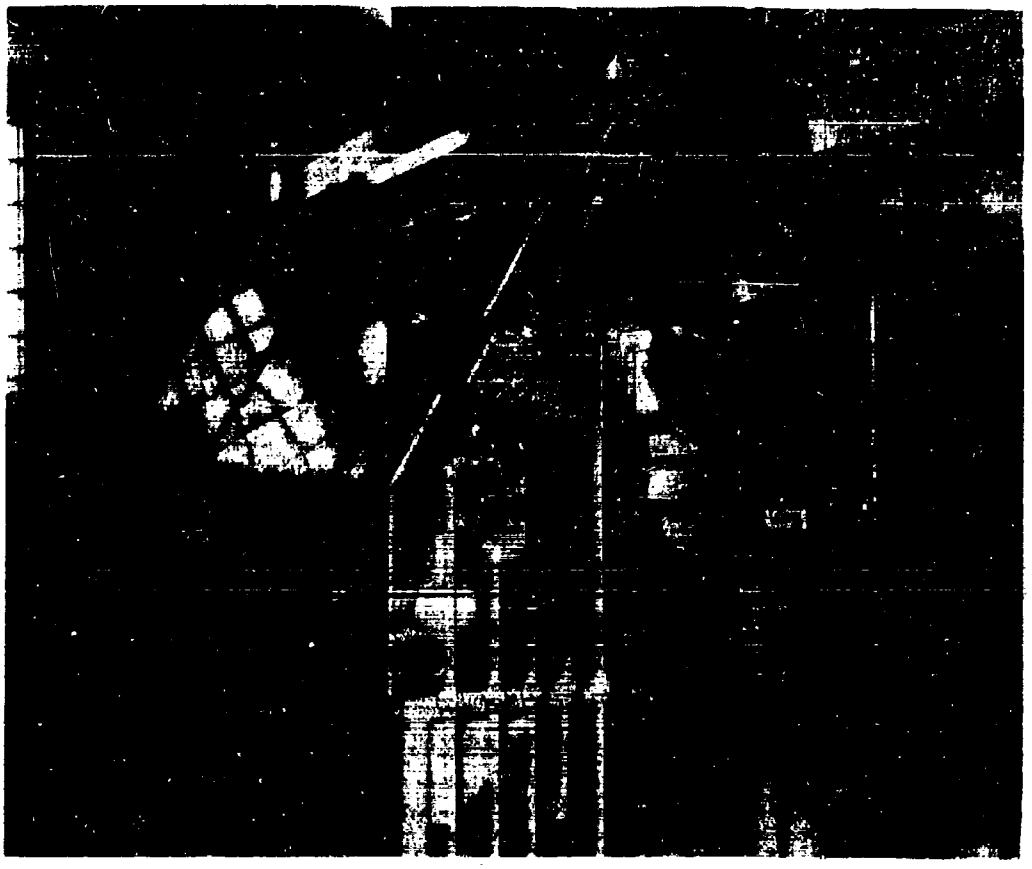


Figure 13. Ventilated Safety Cabinet. (FD Neg C-4172)

B. GROSS PATHOLOGICAL, BACTERIOLOGICAL, OR SEROLOGICAL DIAGNOSTIC METHODS FOR INDUCED INFECTIONS

Standard necropsy procedures are used to attain uniformity in selected techniques and accuracy in diagnosis. Details of a recommended necropsy technique can be obtained from the training manual prepared by the Armed Forces Institute of Pathology.[162]

Some of the general diagnostic procedures used for determining positive animal responses to induced air-borne infections are summarized below. The following descriptions are considered to be illustrative rather than a definitive and rigid protocol for air-borne disease diagnosis. Surprisingly enough, frank primary pneumonias appear to be exceptions instead of the rule in animals challenged via the respiratory route.

1. General Observations

Record weight and observe condition of animals as received from animal holding area. Note condition of coat, discharge from body orifices, and signs of emaciation and dehydration. Check eyes for conjunctivitis, skin of face and axillary and inguinal areas for erythema and petechiae.

2. Gross Examination

The ventral skin is slit from the mandibular symphyses to the pubis. The subcutis is observed and the cervical, axillary, and inguinal lymph nodes are exposed for examination. The abdominal and thoracic cavities are opened and the lungs, heart, tracheobronchial lymph nodes, pericardium, and mediastinum are examined. In the abdominal cavity, the liver, spleen, stomach, intestines, peritoneum, adrenals, kidneys, bladder, and genitalia are examined. As examples, the diagnostic criteria for several aerogenically induced diseases follow.

a. Bacillus anthracis

Animal Holding Period: Ten days for guinea pigs and monkeys. An additional 3 to 5 per cent mortality may occur in monkeys if the surviving animals are held for up to six months.

Positive Response Criteria: Death of animal with positive spleen pathology or observation of anthrax organisms in tissue or blood smears.

Confirmatory Gross Pathology: The characteristic finding is an enlarged dark-colored spleen of jam-like consistency. Blood is not clotted. Lung pathology ranges from hyperemia and pneumonitis to severe edema and gross hemorrhagia. In monkeys, however, gross changes in the lungs are usually minimal.

Bacteriology and Serology: If pathology is doubtful, smears of heart blood and cut surfaces of spleen and liver are prepared and stained with Giemsa's. Observation of at least one or two typical anthrax bacilli is considered a positive diagnosis. Anthrax colonies, after culture on tryptose agar for 24 hours, appear dry, opaque, and raised with irregular edges. Plates prepared from tissue smears will often contain solid bands of confluent growth having a ground-glass appearance. No serological tests are routinely used. We have been successful in the use of a "reversed Ascoli precipitin" test. By preparing the antigen from spleens of monkeys dead from anthrax, we obtain positive precipitin bands in capillary tubes with serum obtained from aerosol-exposed monkeys. Appropriate controls are necessary with this method.

b. Brucella spp.

Animal Holding Period: 28 days for guinea pigs.

Positive Response Criteria: Animal sacrifice 28 days after exposure; evidence of spleen pathology and recovery of organism with serological confirmation.

Confirmatory Gross Pathology: Spleen is enlarged and dark because of hemorrhagia. Numerous focal necrotic lesions observed. Lymph nodes usually enlarged. Lungs show no characteristic findings.

Bacteriology and Serology: Cultures are made of spleen, liver, and heart blood on tryptose agar. Brucella colonies are seen after 48 to 72 hours' incubation as small, circular, convex, opalescent colonies and are identified by a slide agglutination test with antiserum. The above procedures are followed if animals die during the incubation period. During the 28-day holding period, guinea pig mortality from brucellosis may range from 1 to 30 per cent, dependi g on the particular test.

c. Coccidioides immitis

Animal Holding Period: 30 days for guinea pigs; 90 days for monkeys.

Positive Response Criteria: Recovery of fungus from culture of necropsy material or demonstration of spherules in stained tissue sections is required. Positive serology and skin test conversion also are considered as response indicators.

Confirmatory Gross Pathology: Positive findings usually are confined to the lungs, which show bosselated surfaces, nodule formation, and frank consolidation. Nodules may be in other organs if the disease was or had been disseminated.

Bacteriology and Serology: Tissues are smeared on Sabouraud's dextrose agar containing cycloheximide and chloramphenicol to inhibit bacteria. At 48 hours, colonies are smooth and waxy; after 96 hours' incubation at 37°C, they are large and grayish brown with aerial mycelia. Impression smears made from nodular tissues can be examined without specific staining for spherules. For easier identification of fungal structures, Giemsa, Gomori silver methenamine, and periodic acid-Schiff stains can be used.

Progress of the disease in animals can be followed by coccidioidin skin tests and development of complement-fixing antibodies. A progressively rising titer indicates dissemination and concomitantly a poor prognosis.[183]

d. Diplococcus pneumoniae (Lobar Pneumonia)

Animal Holding Period: 60 days for monkeys.

Positive Response Criteria: Death of animal. Observable clinical symptoms include frequent shivering, general febrile disturbance indicated by increased temperature and heightened pulse, short and gasping breath, irritable cough, and eyes hot and dry. Clinical symptoms may be minimal even in very sick animals, however, and prostration and difficult breathing may be the only indications.

Confirmatory Gross Pathology: Hepatization of affected lobes of lung. Consolidated areas may appear dark red or gray, red occurring in the earlier and gray in the later stages of the disease. Cut surface appears moist, granular with a liver-like consistency. Swelling of liver and spleen as evidenced by rounded edges may be apparent.

Bacteriology and Serology: Inoculate blood agar plates and incubate 24 to 48 hours at 37°C. Colonies are smooth, glistening, transparent, dome-shaped, and are surrounded by a hemolysis. D. pneumoniae may be differentiated from a streptococci by the bile-solubility test and the Quellung reaction.

e. Monkey B Virus

Animal Holding Period: Rabbits, 15 days; monkeys, 30 days.

Positive Response Criteria: Death of animal; significant increases in neutralizing antibodies in surviving monkeys. Hyperesthesia, torticollis, labored breathing, salivation, ocular and nasal discharge, conjunctivitis, and corneal opacity usually occur in rabbits about 48 hours preceding death. Inactivity, loss of appetite, and increase in respiration rate may occur in monkeys prior to death.

Confirmatory Gross Pathology: Moderate to extensive lung consolidation is consistently seen as the only gross abnormality in either animal following death; edema fluid may or may not be present.

Virology and Serology: Intracerebral inoculation of normal rabbits with suspensions of lung, brain, and conjunctiva from rabbits dead of B virus produces a typical B virus ascending paralysis resulting in death in 7 to 12 days. B virus recovered from monkey tissues can be identified by neutralization with B virus antiserum prepared in rabbits. Tissues from the lung, salivary gland, spleen, axillary lymph node, spinal cord, and buccal mucosa of monkeys dead of B virus will produce cytopathogenesis in epithelial cells of rabbit kidney.

f. Mycobacterium tuberculosis

Animal Holding Period: 60 to 300 days for monkeys.

Positive Response Criteria: Death of animal. A positive tuberculin test usually is obtained in previously negative animals. No readily observable clinical symptoms may appear until the disease is far advanced or, in fact, prior to death. Decrease in activity and alertness may be the only apparent symptom.

Confirmatory Gross Pathology: Intrathoracic primary lesions. Bronchial nodes greatly enlarged and caseous. Lesions may be seen as miliary tubercles, caseous coagulation with central necrosis, and confluent granulomas in one or more lobes of the lungs. Cavitation within caseous areas is common.[184] In animals that do not succumb, primary lesions may be too small to detect except histopathologically.

Bacteriology and Serology: Acid-fast straining of lung (tissue) smear to demonstrate acid-fast tubercle bacilli. For culturing, inoculate glucose-cysteine-blood-agar plates or membrane filters placed on this medium and incubate for three to four days at 37°C. Examine under dissecting microscope at 10X; colonies are small, white, and heaped in appearance. Virulence may be confirmed tentatively with the neutral red test. Inoculate tuberculin-negative young guinea pigs subcutaneously in the groin or intramuscularly in the thigh. Tuberculin-test guinea pigs at end of four weeks.

g. Pasteurella pestis

Animal Holding Period: 15 days for guinea pigs.

Positive Response Criteria: Death of animal with recovery of P. pestis from animal tissues.

Confirmatory Gross Pathology: There are no particularly pathogenic findings but the lymph nodes, especially the axillary nodes, are

greatly enlarged with necrotic hemorrhagic involvement. Lungs show moderate petechiation and even hemorrhage.

Bacteriology and Serology: Spleen, liver, and heart blood are cultured on blood-agar base media. After 48 hours, colonies appear smooth, glistening, circular, and 0.4 to 0.9 mm in diameter. Cultures are confirmed by tube or slide agglutination tests.

h. Pasteurella tularensis

Animal Holding Period: 10 days for guinea pigs; 30 days for monkeys.

Positive Response Criteria: Death of animal or immunological response (agglutinins) in surviving monkeys. Guinea pigs surviving the 10-day holding period do not exhibit detectable bacteriological, gross pathological, or immunogenic changes. Surviving monkeys may demonstrate agglutinin or precipitin titers.

Confirmatory Gross Pathology: The pathogonomic feature is focal necrotic lesions in the spleen. Small (two to three mm) foci are scattered throughout the usually enlarged and friable spleen, with or without large brownish areas of necrosis. The lungs are moderately to severely affected; changes range from pneumonitis to small miliary nodules to large lesions with caseation necrosis. Petechiation to hemorrhagia may be seen in most organs.

Bacteriology and Serology: Cultures of spleen, liver, and heart blood are prepared on glucose-cysteine-blood-agar plates if pathology is negative or doubtful. Slide or tube agglutinations are used for species confirmation of bacterial colonies appearing on culture plates.

C. USE OF FLUORESCENT ANTIBODY TECHNIQUE FOR DISEASE PATHOGENESIS STUDY

The fluorescent antibody (FA) technique basically is a conventional antigen-antibody procedure with the innovation of labeling the antibody with a dye that fluoresces under an appropriate lighting system. Its application in histopathology probably was fathered by the work of Coons and associates,[165] who in their second publication[166] showed that soluble pneumococcal polysaccharide could be stained in sections of tissue from infected mice. Within the past decade, voluminous studies have been published, indicating the practicality of this method for studies in pathogenesis and in diagnostic microbiology. The application of the FA technique for identification purposes in histology or microbiology is reviewed in Public Health Service Publication 729.[167] Described are FA diagnostic procedures for the fields of parasitology, bacteriology, mycology, virology, and rickettsiology. Tissues containing soluble antigens, bacteria, viruses, or viral antigens usually can be stained directly with FA techniques.

The direct staining method is the simplest form of the many possible variations. It was used by White and Blundell,[168] who demonstrated P. tularensis in formalin-fixed tissue sections of lungs of monkeys infected aerogenically. Intracellular forms of the bacterium were seen in macrophages of the lung and in Kupffer cells from the liver. The publications of McGavran et al[169] and White et al[48] illustrate the type of information obtainable with the FA technique. They studied the morphological and immunohistochemical changes in cynomolgus monkeys intracutaneously or aerogenically vaccinated with an attenuated strain of P. tularensis. Tissue changes were compared in both challenge groups of monkeys. It was possible to demonstrate not only tularemia organisms in the tissues, but also when anti-tularensis γ-globulin (ATGG) first appeared in plasma cell precursors in the lungs, regional lymph nodes, liver, or site of dermal vaccination. FA staining showed that the respiratory bronchiole was the site of deposition and of the initial inflammatory response when the live vaccine strain was inhaled. These data demonstrate not only the usefulness of the FA technique as a valuable tool in charting the course of induced air-borne infections, but also its potential for studying types of cell involved in the defense against invading infectious organisms.

D. VACCINATION BY THE AEROGENIC ROUTE

One practical outgrowth of experimental aerobiological research is the increased interest in the use of aerosols for vaccination. Hitchner and Reising[170] immunized chicken flocks by exposing them to atomized live Newcastle disease virus (B_1 strain). Gorham et al[171,172] immunized ferrets and minks against distemper by allowing the animals to inhale aerosols of egg-adapted virus. Aerogenic vaccination has been considered and practiced by Russian investigators, as indicated in the reports of Aleksandrov et al.[173] They described the effectiveness of attenuated strains of plague, anthrax, tularemia, and brucellosis organisms in animals and man. They also described experiments of mass aerogenic vaccination of people against anthrax.[174] Aerogenic vaccination of monkeys has been reported by Eigelsbach et al.[175] In their studies one group of rhesus monkeys received inhaled doses of 10 viable organisms of a live vaccine strain (LVS) of P. tularensis; another group of monkeys was injected intracutaneously with LVS at a similar dose level. Aerogenic vaccination provided greater immunity to subsequent respiratory challenge with a virulent organism than did vaccination by the dermal route. Higher antibody levels also were seen in the monkeys exposed by the respiratory route. Subsequent tests with large numbers of guinea pigs confirmed the results obtained with primates. Middlebrook[85] used the air-borne route to immunize against experimental tuberculosis. He showed that inhalation by guinea pigs of very small numbers of viable units of BCG organisms resulted in infection and development of dermal tuberculosis hypersensitivity and resistance to subsequent challenge with virulent tubercle bacilli. In some limited experimentation with rabbits and mice, however, he was unable to obtain evidence of aerogenic immunity.

It can be concluded that vaccination by inhalation is an effective means for producing adequate immunization without undue systemic reaction. That this also may be a very convenient method for immunizing large groups of people has been indicated in the referenced reports from Russia. It is noteworthy that Rosenthal (as cited by Middlebrook[65]) has aerogenically vaccinated human subjects with BCG vaccine as they were seated in a 467-foot-square chamber into which the vaccine was aerosolized.

IX. LABORATORY DESIGN PRINCIPLES

A. BUILDING LOCATION, LAYOUT, AND CONSTRUCTION MATERIALS

If possible, the facility for infectious aerobiological research should consist of a separate building not used for classrooms or noninfectious work, and removed from public gathering places. If the infectious unit is part of a larger building space, additional emphasis should be placed on containment features and the area selected should at least be in a remote or uncongested part of the building.

Wedum et al[176] discuss the general design requirements for infectious disease laboratories and present typical layout plans for conversion of existing laboratory areas. A more comprehensive document dealing with architectural consideration for all types of animal facilities is the "Proceedings of the Symposium on Research Animal Housing" sponsored by the National Academy of Sciences (see Hill, 1963).[177] Another useful document is the "Guide for Laboratory Animal Facilities and Care," published by the Public Health Service.[178]

In general, the types of areas required for an adequate experimental unit are: (a) clean and contaminated change rooms with showers, (b) UV air locks, (c) laboratory rooms, (d) aerosol test rooms, (e) infectious-animal holding rooms, (f) preparation and dishwashing rooms, and (g) storage rooms. According to the animals used, their source, and other factors, a clean-animal holding room or an area for at least overnight quarantine is desirable. Space for animal holding is more often underestimated than overestimated.

In selecting construction materials and layout arrangements, the prime aim should be to achieve maximum isolation and containment without undue inconvenience. The proper layout will allow smooth flow of traffic and materials along a clean-to-contaminated axis and provide efficient means for preventing the escape of infectious organisms. The most important means of obtaining unit isolation of areas is through the combined use of air pressure balance, filtration of nonrecirculating exhaust air, and ultraviolet air locks and door barriers. Generous use of sight windows and speaking diaphragms between areas is recommended.

It is especially important that finishes and paints provide crack- and crevice-free surfaces and have maximum resistance to the liquid decontaminants planned for use. Use of tile, special cement, or nonpenetrable finish over floors is a necessity, especially in washrooms or animal rooms where acid or corrosive fluids are used.

B. VENTILATION AND AIR TREATMENT

Air supply and exhaust systems are essential to maintain proper air flows and pressures within the infectious unit. No air should be recirculated and it is generally advisable to filter the air exhausted from rooms and cabinets. Although a variety of recommended ventilation rates will be found, 10 changes per hour for laboratory areas and 15 for animal rooms usually are satisfactory except where more air is needed because of the heat load. Air conditioning is desirable to prevent disturbance of the air balance by the opening of doors and windows during warm weather. Humidity requirements are dictated by what is considered reasonable for the comfort of man and animals, but a humidity of at least 50 per cent should be achieved.

Air filters with efficiencies of 90 to 99 per cent for small particulates are acceptable for building exhaust air, provided primary filtration or incineration is used at the site of particularly hazardous operations such as aerosol vessels, ventilated safety cabinets, and ventilated animal cages. A review of methods of air filtration of microbial particles is available as an aid in selecting suitable types of air filters.[179] The most useful high-efficiency filter (greater than 90 per cent retention of 0.3-micron particles) is that known as 50FG glass fiber. No combustible filter material should be used, especially if filter boxes are located in attics or areas where flammable material is present.

Incineration is the preferred method for treating air from contaminated aerosol vessels, but this should be preceded by a microbial filter to assure containment in case the incinerator fails. Tests on a 75-cfm electric incinerator have shown that treatment at 575°F for three seconds is sufficient to sterilize air heavily laden with bacteria spores.[180] A portable electric incinerator has been described for very small amounts (1 cfm) of exhaust air.[181] Another device used to sterilize air volumes up to 10 cfm employs UV irradiation.[182] For large amounts of air, oil-fired or gas-fired incinerators are available.[183]

C. SEWAGE TREATMENT

If internal containment provisions, such as autoclaving infected cages and debris, and sterilizing wash water from aerosol vessels, do not provide adequate assurance that infectious forms will not leave the building via the discharged liquid wastes, a mechanical system for treating such material should be installed. This involves draining the liquids through all-welded metal lines to a blowcase or tank where heat is applied either continuously or on a batch basis. If pathogenic spores are not a consideration, a low-pressure pasteurization system may be sufficient (200°F for 30 seconds). If sterility is needed, the effluents should be treated with steam under pressure for 10 minutes at 260°F.

D. STERILIZATION AND DISINFECTION PROCEDURES

Too often, in the infectious disease laboratory, routine sterilization and disinfection procedures are applied in a haphazard and ineffective manner. This is often due to lack of understanding of the limitations of various methods of decontamination. It is beyond the scope of this paper to discuss the subject in detail. To expedite efficiency in the use of these procedures in infectious laboratories, it is important that at least one member of the staff develops a personal interest in the subject.

The principal means of sterilization or inactivation of microorganisms are (a) heat, (b) liquid decontaminants, (c) vapors and gases, and (d) radiation. Heat is the most effective method and should be used whenever possible. Of the hundreds of liquid decontaminants available, none is ideal. The efficiency of inactivation depends upon method of application, temperature, exposure time, pH, presence of organic matter, and many other factors. Moreover, the inactivation of viruses and rickettsiae with chemical solutions presents particular problems of detection and assay.

A bibliography of the many reports on gaseous disinfectants is available.[184] Formaldehyde, peracetic acid, β-propiolactone, and ethylene oxide are the most commonly used of the vapor and gaseous disinfectants. The first three are suitable for decontaminating rooms and aerosol vessels, although the persistence of formaldehyde and the corrosiveness of peracetic acid must be considered in practical application. Under controlled conditions, such as in an autoclave, ethylene oxide gas mixtures are effective sterilizing agents, convenient to use, versatile, and noncorrosive.[185] Even more than with liquid germicides, the use of gaseous disinfectants requires a complete understanding of all aspects of their use. Practical methods for steam and chemical sterilization have been discussed by Glick et al.[84]

In specific applications, germicidal ultraviolet radiation at 2537 angstroms is an effective means of decontaminanting air and surfaces. It is useful in air locks and door barriers used to separate laboratory areas of different risk level,[176] and as a germicidal barrier radiating across the tops of animal cages to reduce cross infection.[150] Other laboratory applications have been described.[186,182,187]

Table IV lists the criteria for use of the germicidal substances we have found most useful in aerobiological research with infectious agents other than viruses and rickettsiae. With the latter agents much more information is needed.

TABLE IV. RECOMMENDED CONDITIONS FOR USE OF COMMON GERMICIDAL SUBSTANCES AT ROOM TEMPERATURE (25°C)

Germicide	Concentration and Exposure Time for Typical Classes of Microorganisms			
	Vegetative Bacteria	Bacterial Spores	Fungi	Bacterial Toxins
Phenol	5% (5 min)	NR[a]	5% (15 min)	NR
Lysol	2% (5 min)	NR	3% (15 min)	NR
Quaternary ammonium compound (Roccal, Purasan, Hyamine, etc.)	0.1-1.0% (5 min)	NR	NR	NR
Hypochlorites + 1% wetting agent (Naccanol, etc.)	200-1000 ppm (1 min)	500-5000 ppm (5 min)	2000 ppm (10 min)	NR
Caustic sodium hydroxide	2% (15 min)	5% (30 min)	10% (30 min)	5% soln. (pH 11.5) (15 min)
Formalin (37% HCHO)	5% soln. (10 min)	10% soln. (10 min)	5% soln. (10 min)	5% soln. (10 min)
Steam formaldehyde vapor (closed areas)	1 ml/ft^3 in air with RH[b] above 80% (30 min)			NR
β-Propiolactone vapor	200 mg/ft^3 in air with RH above 80% (30 min)			NR
Ethylene oxide gas	300 mg/liter (8-16 hr)			NR

a. NR = not recommended.
b. RH = relative humidity.

X. SAFETY MANAGEMENT IN INFECTIOUS DISEASE LABORATORIES

Administrative duties of laboratory managers include a number of functions such as programming of experiments, providing safety regulations, keeping records of accidents, and selection and training of personnel.

A. SELECTION OF PERSONNEL

Physical standards for employees should be high; employment in an infectious unit would be inadvisable for persons with (a) pulmonary disease such as tuberculosis; (b) cardiovascular disease such as rheumatic heart disease, coronary occlusion, or recurrent angina; (c) hepatitis; (d) pronounced cutaneous or respiratory allergy; (e) epilepsy; (f) hernia requiring a truss; or (g) a history of schizophrenia or severe psychoneurosis. In addition, objections to being immunized or having blood samples taken to determine probable resistance to infection, or inability to take daily shower baths, should be considered when interviewing prospective employees. In general, persons with a history of known chronic illness, either mental or physical, should be carefully screened before employment. Other factors of obvious importance should be considered by the proper medical authority. These might deal, for example, with the employment of pregnant women in infectious animal units or persons of certain races for work with agents to which they are especially susceptible.

B. TRAINING OF PERSONNEL

The most important consideration in training is adequate communication to assure that employees are taught the safest techniques for handling infectious substances and operating hazardous equipment. According to the size of the laboratory, a training program may be formal or informal, and training may be by groups or individually. In any case, it should be straightforward so that employees clearly understand what can and cannot be done and why. Beyond this, as suggested by Wedum,[188] it is well to emphasize that planning for accident prevention should be a part of every research plan.

Chatigny[25] has discussed the training in "hot lab" techniques given to each new employee at the Naval Biological Laboratory in California. A general outline for education in microbiological laboratory safety is also available.[189] Methods of training can include lectures, demonstrations, motion pictures, and the reading of a portfolio of information on laboratory safety. Three sound and color films of value in regard to safety and technology in aerobiological laboratories are available from the U. S. Public Health Service Film Library: FG-382, Infectious Hazards of Bacteriological Techniques--18 minutes; M-261, Laboratory Methods for Air-Borne Infection, Part I: The Cloud Chamber--30 minutes; M-304, Laboratory Methods for Air-Borne Infection, Part II: The Henderson Apparatus--30 minutes.

C. ACCIDENT REPORTING AND INVESTIGATION

Although the cardinal points of any safety program are education, engineering, and enforcement, specific safety needs and adequate program evaluations are usually available to laboratory management only when there are conscientious reporting and investigation of accidents. Data on all minor accidents and potential exposures to infectious materials as well as events resulting in injury or illness need to be documented and periodically analyzed. If the isolation and containment features in operations are resulting in inapparent infections as indicated by immunological tests or skin test conversions, the laboratory director should be aware of this development and consider its possible consequences. Such a result shows, for example, that infectious organisms are not being completely contained and that a potential exists for active infection among susceptible employees. Even if the director decides that inapparent infections with a certain infectious disease agent can be tolerated, he may wish to examine the situation carefully if new disease agents with more serious debilitating effects are introduced.

The primary purpose of accident investigation is elimination of the causes. With laboratory infections, because of the difficulty in detecting the creation or escape of air-borne infectious organisms, only a minority of tabulated cases have been identified with known or recorded accidents or exposures.[88] The causes of all mechanical- or chemical-caused lost-time injuries can usually be established by adequate reporting and investigation.

Losses, in terms of lost-time injuries or infections, are a result of a series of events that occur in a certain sequence and result in accidents. Accidents are largely preventable by controlling these events. The events themselves are uncovered by prompt reporting and investigation. In establishing the type of information that should be set down on accident report forms or gathered by subsequent investigation, the categories of accident causation data established by the American Standards Association[180] are useful:

(a) The accident agency and agency parts (the object or substance most closely related to the cause)

(b) The accident type

(c) Unsafe mechanical or physical conditions

(d) Unsafe acts

(e) Unsafe personal factors

Determining the causes of accidently acquired infectious diseases, so that steps can be taken to prevent recurrence, frequently requires an epidemiological approach because of the complex interactions of man, animal, agents, and environments that may be involved.

LITERATURE CITED

1. "Conference on airborne infection," McDermott, Walsh (ed.), Bacteriol. Rev. 25, 1961.

2. "Aerobiology," Moulton, F.R. (ed.), Am. Assoc. Advance Science Publication 17, Washington, D. C., 1942.

3. Wells, W.F. "Airborne contagion and air hygiene," 1st Edition, Harvard University Press, Cambridge, Massachusetts, 1955.

4. Langmuir, A.D. "Airborne infection," In: "Rosenau preventive medicine and public health," 8th Edition, Appleton-Century-Crofts, New York, 1956, pp. 152-167.

5. Williams, R.E.O. "Intramural spread of bacteria and viruses in human populations," Ann. Rev. Microbiol. 14:43-64, 1960.

6. Wells, W.F. "Apparatus for the study of the bacterial behavior of air," Am. J. Public Health 23:58-59, 1933.

7. Chapin, C.V. "The sources and modes of infection," 2nd Edition, John Wiley and Sons, New York, 1912.

8. Chope, H.D., and Smillie, W.G. "Airborne infection," J. Ind. Hyg. Toxicol. 18:780-792, 1936.

9. Lurie, M.B. "Airborne contagion of tuberculosis in an animal room," J. Exptl. Med. 51:743-751, 1930.

10. Wells, W.F. "Droplets and droplet nuclei," Am. J. Hyg. 20:611-618, 1934.

11. Riley, R.L. "Airborne pulmonary tuberculosis," Bacteriol. Rev. 25:243-248, 1961.

12. Riley, R.L.; Mille, C.C.; Myka, W.; Weinstock, N.; Storey, P.B.; Sulton, L.V.; Riley, M.C.; and Wells, W.F. "Aerial dissemination of pulmonary tuberculosis: A two year study of contagion in a tuberculosis ward," Am. J. Hyg. 70:185-196, 1959.

13. Albrink, W.S.; Brooks, S.M.; Biron, R.E.; and Kopel, M. "Human inhalation anthrax: A report of three fatal cases," Am. J. Pathol. 36:457-471, 1960.

14. Sulkin, S.E., and Pike, R.M. "Survey of laboratory infections," Am. J. Public Health 41:769-781, 1951.

15. Overholt, E.L.; Tigertt, W.D.; Kadull, P.J.; and Ward, M.R. "An analysis of 42 cases of laboratory-acquired tularemia: Treatment with broad spectrum antibiotic," Am. J. Med. 30:785-806, 1961.

16. McCrumb, F.R., Jr. "Aerosol infection of man with Pasteurella tularensis," Bacteriol. Rev. 25:262-267, 1961.

17. Meyer, K.F. "Pneumonic plague," Bacteriol. Rev. 25:249-261, 1961.

18. Furcolow, M.L., and Ney, P. "Epidemiologic aspects of histoplasmosis," Am. J. Hyg. 65:264-270, 1957.

19. Smith, C.E.; Pappagianis, D.; and Saito, M.T. "The public health significance on coccidiodomycosis," Proc. Symposium on Coccidiodomycosis, U. S. Public Health Service Publication 575:3-9, 1957.

20. Welsh, H.H.; Lennette, E.H.; Abinanti, F.R.; and Winn, J.F. "Airborne transmission of Q fever: The role of parturition in the generation of infective aerosols," Ann. N. Y. Acad. Sci. 70:528-540, 1958.

21. Francis, T., Jr. "Influenza in perspective," In: "International conference on Asian influenza," Am. Rev. Respirat. Diseases 83:98-104, 1961.

22. Robertson, O.H., and Lester, W., Jr. "The lethal effect of triethylene glycol vapor on dried air-borne bacteria," Am. J. Hyg. 53:69-79, 1951.

23. Phillips, G.B., and Hanel, E., Jr. "Use of ultraviolet radiation in microbiological laboratories." U. S. Government Research Reports 34, U. S. Library of Congress, 1960, p. 122.

24. Guyton, H.G.; Buchanan, L.M.; and Lense, F.T. "Evaluation of respiratory protection of contagion masks," Appl. Microbiol. 4:141-143, 1956.

25. Chatigny, M.A. "Protection against infection in the microbiological laboratory: Devices and procedures," In: Advances in Applied Microbiology, 3rd edition, Academic Press, New York, 1961, pp. 131-192.

26. Green, H.L., and Lane, W.R. "Particulate clouds: Dusts, smokes, and mists," D. Van Nostrand Co., Inc., Princeton, New Jersey, 1957.

27. Lowell, F.C., and Schillen, I.W. "Measurement of changes in vital capacity as a means of detecting pulmonary reactions to inhaled aerosolized allergenic extracts in asthmatic subjects." J. Allergy 19:100, 1948.

28. "Behavior of aerosols," Ann. N. Y. Acad. Sci. 105:31, 1963.

29. Public Health Monograph 60, "Sampling microbiological aerosols," Superintendent of Documents, U.S. Government Printing Office, Washington 25, D. C. (U.S. Public Health Service Publication 686, 1959).

30. Glassman, H.N. "Letter to the editor, Microbial aerosols in air-borne infection," Am. J. Public Health 51:1210-1211, 1961.

31. Cardella, M.; Jemski, J.V.; Tonik, E.J.; and Flock, Mary. "Resistance of guinea pigs immunized with botulinum toxoids to aerogenic challenge with toxin," Bacteriol. Proc., 1963, p. 70.

32. Harper, G.J.; Hood, A.M.; and Morton, J.D. "Air-borne microorganisms: A technique for studying their survival," J. Hyg. 56:364-370, 1958.

33. Wolfe, E.K., Jr. "Quantitative characterization of aerosols," Bacteriol. Rev. 25:194-202, 1961.

34. Perkins, W.A., and Vaughan, L.M. "Public health implications of air-borne infection: Physical aspects," Bacteriol. Rev. 25:347-355, 1961.

35. Harper, G.J., and Morton, J.D. "Bacillus subtilis spores labelled with radiophosphorus," J. Gen. Microbiol. 7:98-106, 1952.

36. Miller, W.S.; Scherff, R.A.; Piepoli, C.R.; and Idoine, L.S. "Physical tracers for bacterial aerosols," Appl. Microbiol. 9:248-251, 1961.

37. Orr, C.J., and DallaValle, J.M. "Fine particle measurement," The MacMillan Co., New York, 1959.

38. May, K.R., and Druett, H.A. "The pre-impinger: A selective aerosol sampler," Brit. J. Ind. Med. 10:142-151, 1953.

39. Pilcher, J.M.; Mitchell, R.I.; and Thomas, R.E. "The cascade impactor for particle-size analysis of aerosols," Proc. Forty-Second Annual Meeting, Chemical Specialties Manufacturers Association, Inc., New York, 1955, pp. 74-80.

40. Hatch, T. "Respiratory dust retention and elimination," In: Proc. pneumonoconiosis conference, Johannesburg, Little, Brown and Company, Boston, 1959, pp. 113-132.

41. Landahl, H.D. "On the removal of air-borne droplets by the human respiratory tract: II. The nasal passages." Bull. Math. Biophys. 12:43-56; 161-169, 1950.

42. Casarett, L.J. "Some physical and physiological factors controlling the fate of inhaled substances: II. Retention," Health Phys. 2:379, 1960.

43. Morrow, P.E. "Some physical and physiological factors controlling the fate of inhaled substances: I. Deposition," Health Phys. 2:336, 1960.

44. Goodlow, R.J., and Leonard, F.A. "Viability and infectivity of microorganisms in experimental airborne infection," Bacteriol. Rev. 25:182-187, 1961.

45. Fothergill, L.D. "Symposium on microbial aerosols and respiratory infections," Bacteriol. Rev. 21:249-250, 1957.

46. Langmuir, A.D. "Epidemiology of air-borne infection," Bacteriol. Rev. 25:173-181, 1961.

47. McGavran, M.H.; Beard, C.W.; Berendt, R.F.; and Nakamura, R.M. "The pathogenesis of psittacosis: Serial studies on rhesus monkeys exposed to a small-particle aerosol of the Borg strain," Am. J. Pathol. 40:653-670, 1962.

48. White, J.D.; McGavran, M.H.; Prickett, P.A.; Tulis, J.J.; and Eigelsbach, H.T. "Morphologic and immunohistochemical studies of the pathogenesis of infection and antibody formation subsequent to vaccination of Macaca irus with an attenuated strain of Pasteurella tularensis: II. Aerogenic vaccination," Am. J. Pathol. 41:405-413, 1962.

49. Rosebury, T. "Experimental air-borne infection," The Williams and Wilkins Company, Baltimore, 1947, pp. 54-63.

50. Collison, W.E. "Inhalation therapy technique," Heineman, London, 1935.

51. Griffin, G.W.; Kantzes, H.L.; Ludford, P.M.; and Pelczar, M. J. "Cloud chamber technic for studying aerosols," Appl. Microbiol. 4:17-20, 1956.

52. Ames, A.M., and Nungester, W.J. "The initial distribution of air-borne bacteria in the host," J. Infect. Diseases 84:56-63, 1949.

53. Edward, D.G.F.; Elford, W.J.; and Laidlaw, P.P. "Studies on air-borne virus infections. I. Experimental technique and preliminary observations on influenza and infectious ectromelia," J. Hyg. 43:1-10, 1943.

54. Glover, R.E. "Infection of mice with Mycobacterium tuberculosis (Bovis) by the respiratory route," Brit. J. Exptl. Pathol. 25:141-149, 1944.

55. Loosli, C.G.; Robertson, O.H.; and Puck, T.T. "The production of experimental influenza in mice by inhalation of atmospheres containing influenza virus dispersed as fine droplets," J. Infect. Diseases 72:142-152, 1943.

56. Lange, B., and Koschischian, K.H. "Ueber Versuche, weisse Mäuse durch Einatmung von Krankheitserregern zu infizieren," Z. Hyg. Infektionskrankh 103:569-853, 1924.

57. Martini, E. "Ueber Inhalationspest der Ratten," Z. Hyg. Infektionskrankh 38:332-342, 1901.

58. Buechner, H. "Untersuchungen uber den Durchtritt von Infection serregern durch die Intacte lungenoberflache," Arch. Hyg. (Berlin) 8:144-245, 1888.

59. Young, G.A., Jr.; Zelle, M.R.; and Lincoln, R.E. "Respiratory pathogenicity of Bacillus anthracis spores: I. Methods of study and observations of pathogenesis," J. Infect. Diseases 79:233-246, 1946.

60. Wolochow, H.; Chatigny, M.; and Speck, R.S. "Studies on the experimental epidemiology of respiratory infections: VII. Apparatus for the exposure of monkeys to infectious aerosols," J. Infect. Diseases 100: 48-57; 58-69, 1957.

61. Albrink, W.S., and Goodlow, R.J. "Experimental inhalation anthrax in the chimpanzee," Am. J. Pathol. 35:1055-1065, 1959.

62. Pirsch, G.W.; Day, W.C.; and Pollok, N.L. "Dihydrostreptomycin aerosol therapy for Pasteurella tularensis infected guinea pigs," J. Infect. Diseases 106:231-236, 1960.

63. Weiss, E., and Sigeler, J.C. "A cloud chamber for the uniform air-born inoculation of mice," J. Infect. Diseases 90:13-20, 1952.

64. Middlebrook, G. "An apparatus for air-borne infection in mice," Proc. Soc. Exptl. Biol. Med. 80:105-110, 1952.

65. Middlebrook, G. "Immunological aspects of air-borne infection: Reactions to inhaled antigens," Bacteriol. Rev. 25:331-335, 1961.

66. Gogolak, F.M. "A quantitative study of the infectivity of murine pneumonitis virus in mice in a cloud chamber of improved design," J. Infect. Diseases 92:240-272, 1953.

67. Urban, E.C.J. "Two chambers for use in exposing laboratory animals to aerosols," Arch. Indust. Hyg. Occupat. Med. 9:62-68, 1954.

68. Ferry, R.M.; Brown, W.F.; and Daman, E.B. "Studies on the loss of viability of stored bacterial aerosols: II. Death rates of several non-pathogenic organisms in relation to biological and structural characteristics," J. Hyg. 56:125-150, 1958.

69. Goldberg, L.J.; Watkins, H.M.S.; Boerke, E.E.; and Chatigny, M.A. "The use of a rotating drum for the studies of aerosols over extended periods of time," Am. J. Hyg. 68:85-93, 1958.

70. Ray, F.E., Jr. "A freon-tight chamber for quantitative studies of aerosols of infectious microorganisms," Abst. 136th Meeting Amer. Chem. Soc., 1959, p. 27.

71. Tigertt, W.D., and Benenson, A.S. "Studies on Q fever in man," Trans. Assoc. Am. Physicians 69:90-104, 1956.

72. Tigertt, W.D.; Benenson, A.S.; and Gochenour, W.S. "Air-borne Q fever," Bacteriol. Rev. 25:285-293, 1961.

73. Druett, H.A., and May, K.R. "A wind tunnel for the study of air-borne infections," J. Hyg. 50:60-81, 1952.

74. Henderson, D.W. "An apparatus for the study of air-borne infection," J. Hyg. 50:53-68, 1952.

75. Chappel, A.W. "Animal infectivity of aerosols of monkey B virus," Ann. N. Y. Acad. Sci. 85:931-934, 1960.

76. Roessler, W.G., and Kautter, D.A. "Modifications to the Henderson apparatus for studying air-borne infections: Evaluations using aerosols of Listeria monocytogenes," J. Infect. Diseases 110:17-22, 1962.

77. Saslaw, S.; Eigelsbach, H.T.; Prior, J.A.; Wilson, H.E.; and Carhart, Sally. "Tularemia vaccine study: II. Respiratory challenge," Arch. Internal Med. 107:702-714, 1961.

78. Speck, R.S.; Silverman, M.S.; and Greenman, V. "The behavior of avirulent Klebsiella pneumoniae in the lungs of X-irradiated mice," Bacteriol. Proc., 1959. p. 96.

79. Leif, W.R., and Krueger, A.P. "Studies on the experimental epidemiology of respiratory infections: I. An apparatus for the quantitative study of air-borne respiratory pathogens," J. Infect. Diseases 87:103-116, 1950.

80. Scheckmeister, I.I., and Goldberg, L.J. "Studies on the experimental epidemiology of respiratory infections: II. Observations on the behavior of aerosols of Streptococcus zooepidemicus," J. Infect. Diseases 87:117-127, 1950.

81. Kethley, T.W.; Fincher, E.L.; and Cown, W.B. "The effect of sampling method upon the apparent response of air-borne bacteria to temperature and relative humidity," J. Infect. Diseases 100:97-102, 1957.

82. Webb, S.J. "Factors affecting the viability of air-borne bacteria: II. The effect of chemical additives on the behavior of air-borne cells," Can. J. Microbiol. 6:71-87, 1960.

83. Decker, H.M.; Harstad, J.B.; and Lense, F.T. "Removal of bacteria from air streams by glass fiber filters," Air Pollution Control Assoc. 7:15-16, 1957.

84. Glick, C.A.; Gremillion, G.G.; and Bodmer, G.A. "Practical methods and problems of steam and chemical sterilization," Proc. Animal Care Panel 11:37-44, 1961.

85. Spiner, D.R., and Hoffman, R.K. "Methods of disinfecting large enclosures with Beta-propiolactone vapor," Appl. Microbiol. 8:152-155, 1960.

86. Barrett, J.P., Jr. "Sterilizing agents for lobund flexible film apparatus," Proc. Animal Care Panel 9:127-133, 1959.

87. Jemski, J.V., and Phillips, G.B. "Microbiological safety equipment," Laboratory Animal Care 13:2-12, 1963.

88. Phillips, G.B., and Jemski, J.V. "Biological safety in the animal laboratory," Laboratory Animal Care 13:13-20, 1963.

89. Hatch, T.F. "Distribution and deposition of inhaled particles in respiratory tract," Bacteriol. Rev. 25:237-240, 1961.

90. Zentner, R. "Techniques of aerosol formation," Bacteriol. Rev. 25:188-193, 1961.

91. Wells, W.F. "An apparatus for the study of experimental air-borne diseases," Science 91:172-174, 1940.

92. Loosli, C.G. "An apparatus for nebulizing liquids," Proc. Soc. Exptl. Biol. Med. 57:257-258, 1944.

93. Speck, R.S., and Kelley, J.P. "Active and passive immunity in air-borne Klebsiella pneumoniae infection in mice," Bacteriol. Proc., 1962, p. 92.

94. Dimmock, N.A. "Production of uniform droplets," Nature 166:686-687, 1951.

95. Walton, W.H., and Prewitt, W.L. "The production of sprays and mists of uniform drop sizes by means of spinning-disc type sprayers," Proc. Phys. Soc. (London) 62B:341-350, 1949.

96. May, K.R. "An improved spinning top homogeneous spray apparatus," J. Appl. Phys. 20:932-938, 1949.

97. Albrecht, J. "Quantitative estimation of bacterial aerosols," Z. Aerosol-Forsch. Therapic 4:3-11, 1955.

98. Batchelor, H.W. "Aerosol samplers," Advan. Appl. Microbiol. 2:31-64, 1960.

99. Moulton, S.; Puck, T.T.; and Lemon, H.M. "An apparatus for determination of the bacterial content of air," Science 97:51-52, 1943.

100. Greenberg, L., and Smith, G.W. "A new instrument for sampling aerial dust," U.S. Bureau of Mines Report of Investigation 2392, 1922.

101. Littlefield, J.B.; Feicht, F.L.; and Schrent, H.H. "The Bureau of Mines midget impinger for dust sampling," U.S. Bureau of Mines Report of Investigation 3360, 1937.

102. Cown, W.B.; Kethley, T.W.; and Fincher, E.L. "The critical orifice liquid impinger as a sampler for bacterial aerosols," Appl. Microbiol. 5:119-124, 1957.

103. Tyler, M.E.; Shipe, E.L.; and Painter, R.B. "Bacterial aerosol samplers: I. Development and evaluation of the all-glass impinger; II. Development and evaluation of the Shipe sampler; III. Comparison of biological and physical effects in liquid impinger samplers," Appl. Microbiol. 7:337-362, 1959.

104. Shipe, E.L.; Tyler, M.E.; and Chapman, P.M. "Bacterial aerosol samplers: II. Development and evaluation of the Shipe sampler," Appl. Microbiol. 7:349-354, 1959.

105. May, K.R. "The Cascade Impactor: An instrument for sampling coarse aerosols," J. Sci. Instr. 22:187-195, 1945.

106. Sonkin, L.S. "Application of the cascade impactor to studies of bacterial aerosols," Am. J. Hyg. 51:319-342, 1950.

107. Andersen, A.A. "New sampler for the collecting, sizing, and enumeration of viable air-borne particles," J. Bacteriol. 76:471-484, 1958.

108. Mitchell, R.I., and Pilcher, J.M. "Improved cascade impactor for measuring aerosol particle sizes in air pollutants, commercial aerosols, cigarette smoke," Ind. Eng. Chem. 51:1039-1042, 1959.

109. Malligo, J., and Idoine, L.S. "Single stage impaction device for particle sizing biological aerosols," Appl. Microbiol. 12:32-36, 1964.

110. DuBuy, H.G., and Crisp, L.R. "A sieve device for sampling air-borne microorganisms," Public Health Rept. 59:829-832, 1944.

111. Decker, H.M., and Wilson, M.E. "A slit sampler for collecting airborne microorganisms," Appl. Microbiol. 2:267-269, 1954.

112. First, M.W., and Silverman, L. "Air sampling with membrane filters," A.M.A. Arch. Ind. Hyg. Occup. Med. 7:1-11, 1953.

113. Brown, C.E., and Griffith, W.B. "Syringe device for studies on bacterial and other aerosols," A.M.A. Arch. Indust. Health 18:415-421, 1958.

114. "Laboratory animals: II. Animals for research," Institute of Laboratory Animal Resources Publication 907, National Research Council, Washington, D. C., 1961.

115. Innes, J.R.M.; Coulton, M.W.; Yevech, P.P.; and Smith, C.L. "Lung mites: Pulmonary acariasis as an enzootic disease caused by Pneumonyssus simicola in imported monkeys," Am. J. Pathol. 30:813-835, 1954.

116. Graham, G.L. "Parasitism in monkeys," Ann. N. Y. Acad. Sci. 85:842-860, 1960.

117. Ruch, T.C. "Diseases of laboratory primates," W. B. Saunders Company, Philadelphia, Pennsylvania, 1959.

118. Haberman, R.T., and Williams, F.P. "Diseases seen at necropsy of 708 Macaca mulatta (rhesus monkeys) and Macaca philippinensis (cynomolgus monkeys)," Am. J. Vet. Res. 18:419-426, 1957.

119. Hardy, A.V. "Problems in the control of infectious diseases in a large colony of primates," Proc. Animal Care Panel 5:16-21, 1954.

120. Gay, W.I. "Husbandry practices for the quarantining and conditioning of sub-human primates," Proc. Animal Care Panel 7:83-88, 1957.

121. Young, R.J.; Flemming, B.D.; Benson, R.E.; and Harris, M.D. "Care and management of a Macaca mulatta monkey colony," Proc. Animal Care Panel 7:67-82, 1957.

122. Gisler, D.B.; Benson, R.E.; and Young, R.J. "Colony husbandry of research monkeys," Ann. N. Y. Acad. Sci. 85:758-768, 1960.

123. Sauer, R.H., and Fegley, H.C. "The roles of infectious and noninfectious diseases in monkey health," Ann. N. Y. Acad. Sci. 85:866-888, 1960.

124. "Laboratory animals: I. Guide for the shipment of small laboratory animals," Institute of Laboratory Animal Resources Publication 846, National Research Council, Washington, D. C., 1961.

125. Flynn, R.J. "Studies on the etiology of rats," Proc. Animal Care Panel 9:155, 1959.

126. Druett, H.A.; Henderson, D.W.; Packman, L.; and Peacock, S. "Studies on respiratory infection: I. The influence of particle size on respiratory infection with anthrax spores," J. Hyg. 51:359-371, 1953.

127. Fukui, G.M.; Lawton, W.D.; Janssen, W.A.; and Surgalla, M.J. "Response of guinea pig lungs to in vivo and in vitro cultures of Pasteurella pestis," J. Infect. Diseases 100:103-107, 1957.

128. Speck, R.S., and Wolochow, H. "Studies on the experimental epidemiology of respiratory infections: VIII. Experimental pneumonic plague in Macacus rhesus," J. Infect. Diseases 100:58-69, 1957.

129. Ehrenkranz, N.J., and Meyer, K.F. "Studies on immunization against plague: VIII. Study of three immunizing preparations in protecting primates against pneumonic plague," J. Infect. Diseases 96:138-144, 1955.

130. Jemski, J.V., Kirchheimer, W.F.; Phillips, G.B.; and Maire, L.F. "Tuberculin testing of monkeys experimentally infected with aerosols of tubercle bacilli," Bacteriol. Proc., 1958, p. 79.

131. McCrumb, F.R., Jr.; Larson, A.; and Meyer, K.F. "The chemotherapy of experimental plague in the primate host," J. Infect. Diseases 92:273-287, 1953.

132. Jemski, J.V. "Maintenance of monkeys experimentally infected with organisms infectious for man," Proc. Animal Care Panel 12:89-98, 1962.

133. Finney, D.J. "Statistical Methods in Biological Assay," Charles Griffen and Co., Ltd., London, 1952.

134. Bliss, C.I. "The statistics of bio-assay," In: "Vitamin Methods 2," Academic Press, New York, 1952, pp. 445-620.

135. DeArmon, I.A., Jr., and Lincoln, R.E. "Number of animals required in the bio-assay of pathogens," J. Bacteriol. 78:651-657, 1959.

136. Guyton, A.C. "Measurement of the respiratory volume of laboratory animals," Am. J. Physiol. 150:70-77, 1947.

137. Schlamm, N.A. "Detection of viability in aged or injured Pasteurella pestis," J. Bacteriol. 80:818-822, 1960.

138. Graham, W.R., and Feenstra, E.S. "A program for the development of pathogen-free laboratory animals," Proc. Animal Care Panel 8:54-66, 1958.

139. Druett, H.A.; Robinson, J.M.; Henderson, D.W.; Packman, L.; and Peacock, S. "Studies on respiratory infection: II. The influence of aerosol particle size on infection of the guinea pig with Pasteurella pestis," J. Hyg. 54:37-48, 1956.

140. Phillips, G.B.; Jemski, J.V.; and Brant, H.G. "Cross infection among animals challenged with Bacillus anthracis," J. Infect. Diseases 99:222-226, 1956.

141. Kirchheimer, W.F.; Jemski, J.V.; and Phillips, G.B. "Cross infection among experimental animals by organisms infectious for man," Proc. Animal Care Panel 11:83-92, 1961.

142. Phillips, G.B.; Broadwater, G.C.; Reitman, M.; and Alg, R.L. "Cross infections among brucella infected guinea pigs," J. Infect. Diseases 99:56-59, 1956.

143. Horsfall, F.L., Jr., and Bauer, J.H. "Individual isolation of infected animals in a single room," J. Bacteriol. 40:569-580, 1940.

144. Levine, P.P., and Fabricant, J. "Susceptibility to Newcastle infection of chicks with congenital serum antibodies," Cornell Vet. 40:213-225, 1950.

145. Young, G.A., and Underdahl, N.R. "Isolation units for growing pigs without colostrum," Am. J. Vet. Res. 16:571-574, 1953.

146. Haelterman, E.O. "Practical isolation equipment for baby pigs," Am. J. Vet. Res. 17:129-131, 1956.

147. Dolowy, W.C. "Medical Research Laboratory of the University of Illinois," Proc. Animal Care Panel 11:267-280, 1961.

148. Lind, A. "Danger of T.B. infection to personnel in pathological laboratories," Transactions of the NAPT Commonwealth Chest Conference. National Association for the Prevention of Tuberculosis. Tavistock House North, Tavistock Square, London, W.C.1., England, 1957.

149. Lurie, M.B. "The prevention of natural air-borne contagion of tuberculosis in rabbits by ultraviolet irradiation," J. Exptl. Med. 79:559-572, 1944.

150. Phillips, G.B.; Reitman, M.; Mullican, C.L.; and Gardner, G.D., Jr. "Applications of germicidal ultraviolet in infectious disease laboratories: III. The use of ultraviolet barriers on animal cage racks," Proc. Animal Care Panel 7:235-244, 1957.

151. Gremillion, G.G. "The use of bacteria-tight cabinets in the infectious disease laboratories," Proc. 2nd Sym. on Gnotobiotic Technology, Univ. of Notre Dame Press, Notre Dame, Indiana, 1960, pp. 171-182.

152. Kraft, L.M. "Aerial cross infection of mouse diarrhea," Yale J. Biol. Med. 31:121-137, 1958.

153. Haenni, E.O.; Affens, W.A.; Lento, H.G.; Yoemans, A.H.; and Fulton, R.A. "New nonflammable formulations for sterilizing sensitive materials," Ind. Eng. Chem. 51:685-688, 1959.

154. Guyton, H.G., and Lense, F.T. "Methods for evaluating respiratory protective masks and their component parts," A.M.A. Arch. Ind. Health 14:246-249, 1956.

155. Guyton, H.G., and Decker, H.M. "Respiratory protection provided by five new contagion masks," Appl. Microbiol. 11:66-68, 1963.

156. Fish, C.H., and Spendlove, G.A. "Safety measures in a tuberculosis laboratory," Public Health Rept. 65:466-467, 1950.

157. Black, J.; Lynch, J.M.; and Ladimer, I. "A program for protection of research employees against pathogenic hazards," Public Health Rept. 68:989-992, 1953.

158. Wedum, A.G. "Bacteriological safety," Am. J. Public Health 43:1428-1437, 1953.

159. Lind, A. "Ventilated cabinets in a tuberculosis laboratory," Bull. World Health Organ. 16:448-453, 1957.

160. Couling, C.W., and Rees, R.J.W. "A protective cabinet for the post-mortem examination of infected animals," J. Hyg. 57:407-409, 1959.

161. Blickman, B.I., and Lanahan, T.B. "Ventilated work cabinets reduce lab risks," Safety Maintenance 120:34-36, 1960.

162. AFIP Technical Manual 8-300. "Autopsy manual," Superintendent of Documents, U. S. Government Printing Office, Washington 25, D. C., 1960.

163. Converse, J.L.; Lowe, E.P.; Castleberry, M.W.; Blundell, G.P.; and Besemer, A.R. "Pathogenesis of Coccidioides immitis in monkeys," J. Bacteriol. 83:871-878, 1962.

164. Schmidt, L.H. "Some observations on the unity of simian pulmonary tuberculosis in defining the therapeutic potentialities of isoniazid," Am. Rev. Tuberc. Pulmonary Disease Supplement, August 1956, pp. 138-153.

165. Coons, A.H.; Creech, H.J.; and Jones, R.N. "Immunological properties of an antibody containing a fluorescent group," Proc. Soc. Exptl. Biol. Med. 47:200-202, 1941.

166. Coons, A.H.; Creech, H.J.; Jones, R.N. and Berliner, E. "The demonstration of pneumococcal antigen in tissues by the use of fluorescent antibody," J. Immunol. 45:159-170, 1942.

167. "Fluorescent antibody techniques," Superintendent of Documents, U. S. Government Printing Office, Washington, D. C. (Public Health Service Publication 729, 1960).

168. White, J.D., and Blundell, G.P. "The use of the fluorescent antibody technique for demonstration of Pasteurella tularensis in formalin-fixed tissue," Bacteriol. Proc., 1958, p. 136.

169. McGavran, M.H.; White, J.D.; Eigelsbach, H.T.; and Kerpsack, R.W. "Morphologic and immunohistochemical studies of the pathogenesis of infection and antibody formation subsequent to vaccination of Macaca irus with an attenuated strain of Pasteurella tularensis: I. Intracutaneous vaccination," Am. J. Pathol. 41:259-271, 1962.

170. Hitchner, S.B., and Reising, G. "Flock vaccination for Newcastle disease by atomization of the B strain of virus," Proc. Am. Vet. Med. Assoc. 89:258-264, 1952.

171. Gorham, J.R.; Leader, R.W.; and Gutierrez, B.S. "Distemper immunization of mink by air-borne infection with egg-adapted virus," J. Am. Vet. Med. Assoc. 125:134-136, 1954.

172. Gorham, J.R.; Leader, R.W.; and Gutierrez, J.C. "Distemper immunization of ferrets by nebulization with egg-adapted virus," Science 119: 125-126, 1954.

173. Aleksandrov, N.I.; Gefen, N.E.; Garin, N.S.; Gapochko, K.G.; Daal-Berg, I.I.; and Sergeyev, V.M. "Reactivity and effectiveness of aerogenic vaccination against certain zoonoses," Voenno-Med. Zh. 12: 34-38, 1958.

174. Aleksandrov, N.I.; Gefen, N.E.; Garin, N.S.; Gapochko, K.G.; Sergeyev, V.M.; Smirnov, M.S.; Tamarin, A.L.; and Shliakhov, E.N. "Experiment of mass aerogenic vaccination of people against anthrax," Voenno-Med. Zh. 8:27-32, 1959.

175. Eigelsbach, H.T.; Tulis, J.J.; Overholt, E.L.; and Griffith, W.R. "Aerogenic vaccination against tularemia," Bacteriol. Proc., 1960, p. 89.

176. Wedum, A.G.; Hanel, E., Jr.; Phillips, G.B.; and Miller, O.T. "Laboratory design for study of infectious disease," Am. J. Public Health 46:1102-1113, 1956.

177. "Proceedings of the Symposium on Research Animal Housing," Laboratory Animal Care 13:2:222-467, 1963.

178. "Guide for laboratory animal facilities and care," Superintendent of Documents, U. S. Government Printing Office, Washington 25, D. C. (Public Health Service Publication 1024, 1963).

179. Decker, H.M.; Buchanan, L.M.; Hall, L.B.; and Goddard, K.R. "Air Filtration of Microbial Particles." Superintendent of Documents, U. S. Government Printing Office, Washington 25, D. C. (Public Health Service Publication 953, 1962).

180. Decker, H.M.; Citek, F.J.; Harstad, J.B.; Gross, N.H.; and Piper, F.J. "Time temperature studies of spore penetration through an electric air sterilizer," Appl. Microbiol. 2:33-36, 1954.

181. Gremillion, G.G.; Miller, L.F.; and Bodmer, G.A. "An electric incinerator for sterilization of small volumes of air," Appl. Microbiol. 6:274-276, 1958.

182. Miller, O.T.; Schmitt, R.F.; and Phillips, G.B. "Application of germicidal ultraviolet in infectious disease laboratories: I. Sterilization of small volumes of air by ultraviolet radiation," Am. J. Public Health 45:142-143, 1955.

183. Sharpe, B.M. "Sterilizing germ-contaminated air by direct heating," Indust. Heating 21:745, 1954.

184. Phillips, G.B.; Harris, G.J.; and Jemski, J.V. "Microbiological safety bibliography." Report of Biological Safety Committee, Animal Care Panel, Argonne, Illinois, 1961.

185. Phillips, C.R., and Warshowsky, B. "Chemical disinfectants," Ann. Rev. Microbiol. 12:525-550, 1958.

186. Harstad, J.B.; Decker, H.M.; and Wedum, A.G. "Use of ultraviolet irradiation in a room air conditioner for removal of bacteria," Appl. microbiol. 2:148-151, 1954.

187. Phillips, G.B., and Novak, F.E. "Application of germicidal ultraviolet in infectious disease laboratories: II. An ultraviolet pass-through chamber for disinfecting single sheets of paper," Appl. Microbiol. 4:95-96, 1956.

188. Wedum, A.G. "Policy, responsibility, and practice in laboratory safety," Proc. 2nd Symposium on Gnotobiotic Technology, Univ. of Notre Dame Press, Notre Dame, Indiana, 1959, pp. 105-119.

189. Phillips, G.B. "Safety education for students of microbiology," Am. J. Med. Tech. 28:291-295, 1962.

190. "American recommended practice for compiling industrial accident causes, Pamphlet Z16.2; American Standards Association, New York, 1941.

191. Barnes, J.M. "The development of anthrax following the administration of spores by inhalation," Brit. J. Exptl. Pathol. 28:385-394, 1947.

192. Henderson, D.W.; Peacock, S.; and Belton, F.C. "Observations on the prophylaxis of experimental pulmonary anthrax in the monkey," J. Hyg. 54:28-36, 1956.

193. Ross, J.M. "The pathogenesis of anthrax following the administration of spores by the respiratory route," J. Pathol. Bacteriol. 73:485-494, 1957.

194. Elberg, S.S., and Henderson, D.W. "Respiratory pathogenicity of brucella," J. Infect. Diseases 82:302-306, 1948.

195. Druett, H.A.; Henderson, D.W.; and Peacock, S. "Studies on respiratory infection: III. Experiments with Brucella suis," J. Hyg. 54:49-57, 1956.

196. Larsh, H.W. "Natural and experimental epidemiology of histoplasmosis," Ann. N. Y. Acad. Sci. 89:78-90, 1960.

197. Kautter, D.A.; Silverman, S.J.; and Roessler, W.G. "Studies on the virulence of Listeria monocytogenes via the respiratory route for laboratory animals," Bacteriol. Proc., 1959, p. 95.

198. Lawton, W.D ; Fukui, G.M.; and Surgalla, M.J. "Antigens associated with immunity against plague," Bacteriol. Proc., 1959, p. 88.

199. Harper, G.J., and Morton, J.D. "The respiratory retention of bacterial aerosols: Experiments with radioactive spores," J. Hyg. 51:372-385, 1953.